

Does science leave room for soul?

A new version of the old assertion that science affronts human dignity, published in Britain as a book, may herald an assault on basic science and its sources of support.

Mr George Walden is a Member of Parliament in the British House of Commons who, for two years in the mid-1980s, was the minister in charge of government spending on civil science. In that role, he was articulate and argumentative, demanding of people such as the heads of research councils why they required government funds for this or that purpose, and what the public interest might expect in return. Those questioned usually supposed him to be acting as devil's advocate to gather arguments with which to confront his own paymasters. Not so, it seems.

Last week, in the *Daily Telegraph*, Walden gave the show away. At one stage, he explains, he went to CERN in Geneva during one of the many British re-examinations of continued membership of the European high-energy physics consortium. He now writes of meeting there "soft-spoken zealots . . . with a quasi-mystical belief in what they were doing". He recalls that they offered "the ultimate truth: the constituents of matter" in return for "enough government cash". Walden adds that he has now been helped to understand the origins of his disgust with CERN by a book, *Understanding the Present* by Bryan Appleyard, now published in Britain (Picador, £14.95), whose theme is that science is inimical to the human spirit and its needs. Wistfully, Walden wishes that he had been armed during his visit with the arguments in Appleyard's last chapter, entitled "The humbling of science".

This tale is both part of the explanation why British science was so roughly dealt with in the 1980s — its sponsors were disaffected — and, more ominously, a sign of the way the wind is blowing in the environment of research. After decades in which science and technology (rather than their public administration) have been blamed for nuclear weapons, radioactivity in general, other environmental problems and the ethical dilemmas thrown up by the new biology, they are now being blamed for the complexity and the uncertainty of the modern world. Appleyard's book (to be reviewed in *Nature* by Sir Brian Pippard next week) is both a reflection of this fashion and, because it is both articulate and passionate, a reinforcement of it.

The theme is simple: "Science is not a neutral or innocent commodity, . . . a convenience. Rather, it is spiritually corrosive, burning away ancient authorities and traditions." Dating the beginning of modern science to Galileo's heliocentric challenge, Appleyard takes the succession of later challengers (Newton, Descartes, Darwin, Einstein) as proof

that the assault of science on belief is autonomous and unstoppable. While acknowledging Francis Fukuyama's thesis that science has also made possible the recent triumph of liberal democracy (see *Nature* 356, 271; 1992, Appleyard doubts that "triumph" is the right word: liberal democracies are all too adept at compromising belief (as on the rights and wrongs of abortion, or embryo research).

If Appleyard's book were less likely to be persuasive, it could be dismissed as nostalgic wish-fulfilment. Yet, stripped of its sophistication, that is what it is. It may have been inconvenient that Galileo put the Sun at the centre, but what else, in truth, could he have done? Newton and Descartes may have modelled the Universe as a machine, to the discomfort of many, but what if that (in the widest sense) is what it is? And what if the distinction between people and other animals is indeed less clear-cut than the eighteenth century believed? Ancient authorities and traditions have indeed been burned away in the past four centuries, but that is not the tragedy Appleyard makes it out to be.

Distance in time lends false benignity to certainty in belief. Appleyard mentions that Galileo appeared before the Inquisition, but does not comment on the social iniquity of that device for the reinforcement of belief nor on the civility that has sprung from the more recent erosion of superstition (of which there is still too much). But the baseless and pointless ethnic wars now being fought in Yugoslavia and the *fatwah* uttered by the Moslem hierarchy of Iran against the British author Salman Rushdie are contemporary evidence of where certainty in belief can lead. Appleyard, of course, is asking more modestly that people should have a common sense of purpose inspired by common belief in *something*, but could that persist without illiberal reinforcement?

That is not to deny that there are readings of science's view of the world that evidently fill people such as Appleyard with desolation: on the heels of the model of the Universe as a machine has followed that of plants and animals as DNA machines. But if both (the Universe and living things) are historical accidents, what basis can there be for a collective sense of purpose? St Augustine, helping to administer a system of belief in which the surface of the Earth is but an antechamber to Heaven, and puzzled by people's perverse tenacity in hanging onto life, hit on the explanation that people's regard for their children, and their children's children, is the thread connecting the present to the future. What is wrong with that?



The chattering classes understandably hanker after more. They should reflect — last week's hoopla about the Big Bang notwithstanding — that present models of the Universe and of living things are far from complete. But Appleyard's desolation at the certainty that the Earth will eventually disappear might well be lifted if communication with other planets or even universes were at some stage found possible. (Do not count on that.) Meanwhile, the view that the Earth is a fragile spaceship in the middle of nowhere in particular must surely argue for an understanding of its journey, which is why many have taken understanding as their goal. Appleyard will have none of that.

That is why the book is dangerous. Hand-wringing over the supposed corrosion of belief by science will readily provoke the view that the world would be safer and more congenial if there were less subversion and, so, if there were less science. Already there have been misgivings over human genome sequencing on the grounds that it may yield "more than we want to know". There is even a danger that rationality will be blamed for supposedly novel social ills. Is it coincidence that Mr John Patten, a minister in the present British government (see *Nature* 356, 547; 1992) has been arguing that social violence springs from diminished religious fear of damnation? He, like Walden, shows that there are politicians all too ready to listen to Appleyard's assault on reason. □

Defining misconduct

US Academy offers strict definitions but ducks other urgent issues.

It is the best of times. It is the worst of times. Never before has science been as productive as it is today, and never before has the scientific community been so dominated by concerns about integrity in research and scientific fraud — particularly in the United States. Virtually every US professional organization has examined the issue, thus indirectly lending credence to the notion that the scientific enterprise is rife with miscreants. Last week the US National Academy of Sciences (NAS) had its say. Its report *Responsible Science: Ensuring the Integrity of the Research Process* mirrors the confusion and contention within the academic community where, after a dozen years of debate, some continue resolutely to deny that there is anything to be concerned about, while others argue about procedural solutions.

To its credit, the NAS committee, chaired by Edward E. David, former research head at Exxon and presidential science adviser in the Nixon Administration, is right to call on US science agencies and universities to adopt a strict definition of scientific misconduct: "fabrication, falsification, or plagiarism in proposing, performing, or reporting research". The panel explicitly rejects broader definitions, including that favoured until recently by the US National Institutes of Health (NIH) which included "other serious deviations from accepted research practices" (whatever that may mean) in the definition.

Fairness to the accused and to the reputation of science demands that alleged acts of fraud should be dealt with differently from charges of lack of collegiality in sharing data or credit (see *Nature* 352, 563; 1991). General adoption of the academy's definition, closely matching that proposed recently by an advisory committee to the NIH (see *Nature* 356, 191; 1992), would be in everybody's interests. A strict definition would also pave the way for procedures for handling allegations of fraud that parallel the judicial system: an investigation by independent parties, an opportunity for the accused to confront his or her accusers and the evidence they adduce and a ruling by a duly constituted judging body from which the prosecutors are absent.

So how should allegations of misconduct be handled? The academy committee has plainly, unmistakeably and disappointingly ducked the issue. Were 22 members and two contentious years of study not enough? Has more than a decade of often painful experience at particular institutions, or the draft policies of funding agencies, been in vain? Weakly, the committee says there is not a "sufficient base of institutional experience or consensus . . . within the academic community" for it to say how allegations of misconduct should be handled. If the academy, which supposedly represents the best of the US scientific establishment, cannot render an opinion, to whom should we turn?

The Scientific Integrity Advisory Board, that's who. The committee says the solution lies in yet another committee, to be fashionably comprised of scientists, ethicists, the legally informed and "public figures who are not involved in the scientific enterprise". But this idealized body, independent of any scientific organization, would not deal with actual cases but, instead, would somehow help the community to reach the consensus on misconduct that the academy committee failed to achieve. Yet the committee hopes that the integrity board would be viewed by Congress as a "concrete step" by the scientific community to police its conduct of federally sponsored research. Dutifully, academy president Frank Press says he will convene a committee of the academy to study this academy committee's recommendation for the formation of a committee.

Behind each analysis of scientific conduct lies the hope that emphasis on professional standards will create an environment in which those so under pressure to succeed (or simply so perverse) that they resort to fraud will desist. So arises the question of guidelines for what is now in Americanese called the 'conduct of research'. Four prominent US institutions have recently answered in the affirmative, with guidelines to good research practices: the medical schools at Johns Hopkins and Harvard Universities, the University of Michigan and the intramural research programme at NIH. None of these is so rigid that it could even remotely be considered a threat to creativity. Instead, the rules are both pertinent and practical. Among them, for instance, are these: faculty preceptors should not accept more students in a laboratory than they can supervise personally; editing a student's paper does not justify the title of mentor; honorary co-authorship is deplorable, not to

mention unwise when the data are challenged. And, most important, primary data (in whatever form) should be retained for the active life of the research project or for at least five years, unless there is good reason to the contrary.

Such guidelines are sensible, not onerous. Research institutions that have not yet written their equivalents should promptly do so. Many members of the Academy committee David agree. Nevertheless, collectively the group could not see past the fear that guidelines = detailed rules = interference with research freedom. That reflects the notion, no doubt once true, that proper research behaviour is learned by some form of osmosis at the preceptor's knee and that fraud is found out in attempts at replication. The facts now suggest otherwise; whistleblowers are more prominent than replicators, for example.

What has gone wrong? Academy president Frank Press recently noted that, from Archimedes to Bacon to this century (but he may have overlooked the cloud over Ptolemy's calculations), the scientific method has been self-correcting, but is no longer so. "Science is too big, too complex." Indeed, which is why research institutions need to pay attention not only to fraud but also to lesser but more common forms of undesirable behaviour, fraud's precursors. The Academy's report recognizes this but fails to recommend universal remedies beyond 'education' and local action.

The decline in standards of behaviour in science is most notable at the margins. Always competitive, the endeavour now is more so than ever, sometimes viciously so. Often, competition for has been replaced by competition for money, especially in the biotechnology industry. The idea of a senior scientist working intimately with a handful of students has been relegated to history as laboratories with 50, 60 or more members became the norm. Difficult as it may be to accept, scientific etiquette must now be taught. One academy committee member teaching such a course says he begins by telling students that it is not ethical to 'lie' about data, an "idea some of them never thought of".

That is another reason why the report of the academy committee is such a disappointment — it seems unaware of the realities of the laboratory. By all accounts, the group nearly disbanded after its first meeting because many of its members objected to the idea of a study of scientific misconduct on the grounds that it could only make the problem look worse than it is. The same notion is said to have reared its befuddled head when *Responsible Science* was sent out for peer review. It is, perhaps, remarkable that the report came out at all. But it is ultimately a disservice to perpetuate the idea that the issues of professional behaviour are so complex that even the best minds in science cannot deal with them.

The implementation of procedures for investigating fraud requires judgement and latitude for extenuating circumstances in any given case. It is true that rigid rules about such matters as data retention and sharing reagents would do more harm than good. But the essentials are not complex at all — a truth the research community should be able to accept. □

Big Bang brouhaha

Caution in celebrating last week's announcement of anisotropies in the microwave background would be wise.

If ever a practical demonstration was needed that the whole world is agog to know how the Universe is constructed, last week should have provided it. Hardly a general newspaper in the Western world failed to carry a report on its first page announcing the discovery of a large-scale structure in the pattern of the microwave background radiation on the two-dimensional surface of the sky. (*Nature's* own version, by Professor Joseph Silk, appears on page 741.)

Evidently the microwave satellite COBE is every bit the instrument it was designed to be. But the simple conclusion, that the data so far authenticated are consistent with the doctrine of the Big Bang, has been amplified in newspapers and broadcasts into proof that "we now know" how the Universe began. This is a cause of some alarm.

The search for anisotropies in the microwave background radiation is not a simple matter. People have been looking for variations in the apparent temperature of different patches of the sky of the order of ten millionths of a Kelvin. That they have found them at all is a testament to the power of modern instrumentation. Silk explains how the data gathered have been subjected to the most rigorous checking for consistency, suggesting that the numbers at least can be believed. That the amplitude of the fluctuations observed is consistent with at least some models of galaxy formation is a relief more than a surprise, but the true test will come when other instruments are able to provide measurements of comparable sensitivity on smaller angular scales, where different cosmological theories are more sharply distinguished.

Meanwhile, it is important that the doctrine of the Big Bang is not itself a simple matter. Its origin is supposed to have been the explosion from zero volume at zero time of a corpuscle of energy equivalent to the mass and radiation that now constitute the Universe. Fluctuations have been an intellectual problem for decades; without them, galaxies could not have formed, but there are few physical arguments as to why they should have been present at early times, and even fewer that suggest one spectrum of fluctuations over another. Cosmologists have filled their theoretical universes with some kind of invisible dark matter, to help galaxies form, and many look to inflation — a phase of early exponential — to provide the fluctuations that set the whole process in motion. But for neither dark matter nor inflation is there true independent support, outside the cosmological arena for which they were invented.

Nobody should be surprised, therefore, if the handful of those who reject the Big Bang claim the new data as support for their theories also. It is more relevant that all theories can at last be checked against real data. That will take some time. But it may no longer be the case, as it was some time ago, that Sir Hermann Bondi could begin a public lecture with the observation that "the data in cosmology are so likely to be wrong that I propose to ignore them". □

US leans on Russia to drop rocket agreement

■ US fears technology could be used as weapon
■ Indian officials decry "brazen interference"

New Delhi

PRESSURE from the United States has led Russia to pull back from its agreement to help India to build a rocket to launch communications satellites. The decision could delay or weaken India's effort to carry out independent research in space.

Russian officials have said that the United States has threatened privately to impose trade sanctions if an agreement signed in January 1991 between Glavkosmos, the then Soviet space agency, and the Indian Space Research Organization (ISRO) is not cancelled. The \$80-million contract would have supplied India with a liquid-fuel rocket engine and related technology for the second stage of a geostationary launch vehicle. The rocket is scheduled for launch in 1995.

The dispute involves a policy adopted in 1987 by the industrialized nations to limit the proliferation of missiles capable of delivering nuclear weapons. Although its guidelines state that they "are not designed to impede national space programmes", the technology covered by the Russian-Indian agreement falls within the list of prohibited items. The Soviet Union agreed in 1990 to abide by the terms of the agreement, called the Missile Technology Control Regime (MTCR).

Although the US State Department would not confirm or deny reports that it had threatened to impose trade sanctions if the Russians tried to carry out the contract, a department official said last week that the US government "is opposed to the transfer of such subsystems, and the fact that they are intended to be used for peaceful purposes is not relevant". Indian authorities point out that it takes 90 days to fuel the Russian rocket engine, precluding its use as a military weapon, but the US official says that "any liquid fuel rocket stage that is capable of being used as a space vehicle falls under US export control laws, which are consistent with the MTCR guidelines".

The feud has left Indian officials in a quandary. Work on the design of the launch vehicle was stopped last year on the assumption that the Russian engine would be used. All other stages of the vehicle have been designed and tested, and ISRO says that the programme will be delayed for several years if India has

to develop the engine on its own. ISRO rejected an offer two years ago by Arianespace of France because its price — \$230 million — was thought to be too high.

The Indian prime minister, P.V. Narasimha Rao, said last week that the Russians had not scrapped the deal but were only suggesting "a pause" while they sought further assurance that India would not use the technology to make

DEEP-SEA DRILLING

Japan floats big drilling plans

Tokyo

JAPAN sees deep-sea drilling as its next chance to launch a big international science project. The Science and Technology Agency held a symposium in Tokyo last week to promote its plan to build a huge high-technology deep-sea drilling ship that will dwarf *JOIDES Resolution*, the US ship currently used in the international Ocean Drilling Program (ODP).

The agency's plan calls for a ship of 15,000 tons (gross) — nearly twice the size of *JOIDES Resolution* — to be built by 1998 at a cost of \$300-400 million. In an improvement on the *JOIDES Resolution*, the new ship would be equipped with a riser pipe up to 4 km in length encasing the drill pipe to allow flushing of the drill hole with mud without contaminating the core samples. The agency also plans to develop temperature-resistant drill bits to allow drilling in hot rocks — for example, mid-ocean ridge crests — at temperatures up to 350-400 °C, twice the present limit.

The drilling programme is scheduled to end in 2003 but is expected to be extended to 1998 because of a favourable review by the US National Academy of Sciences and promises of further support from participating nations. But the future of the programme beyond 2003 is uncertain. And so the Japanese ship if built will come at a perfect time, says D. James Baker, president of Joint Oceanographic Institutions Inc., the prime contractor of ODP, who gave a presentation at the Tokyo symposium. Nevertheless, US researchers are anxious that any Japanese vessel should be focused on science, rather than drilling technology. Officials at the US National Science Foundation are concerned that Japan's quest for energy independence may turn the ship into

missiles. The chairman of ISRO, U.R. Rao, returned from Moscow saying that there were no "insurmountable difficulties" in adopting a new contract that would not violate the non-proliferation guidelines but he declined to describe the changes being sought by Russian officials.

The problem came to light two weeks ago when Vitaly Sevastyanov, a cosmonaut and member of the Russian Committee for Foreign Affairs, was quoted by the Russian media as saying that the United States was trying "to drive Russia out of high-technology markets". The reports caused an uproar within the Indian parliament, with opposition members castigating the United States for "brazen interference" in the internal affairs of the two countries.

K.S. Jayaraman

a testbed for oil exploration technology, at the cost of some of its scientific capabilities. There are no current plans for a US replacement vessel, and a proposed Russian ship is still in question given the economic situation.

Originally, the Japanese planned to create a parallel international drilling programme. But now the proposed Japanese drilling ship may be used in a reformed programme that may also include a drilling ship which France plans to build, Baker says. Until now, the United States has been the prime funder of the ocean drilling programme, contributing 50 per cent of the \$40-million annual running costs and 50 per cent of the programme's researchers. But Japan and France may take on leading roles if their plans are realized.

The Japanese programme would be run by the Japan Marine Science and Technology Centre, which the Science and Technology Agency hopes to convert into an international "centre of excellence" with the help of the drilling programme.

But plans by another part of the Science and Technology Agency to launch a huge oceanographic vessel may conflict with the drilling project. The agency's atomic energy bureau hopes to convert the ill-fated nuclear-powered ship *Mutsu* into a giant oceanographic vessel by ripping out the nuclear reactor and replacing it with a diesel engine (*Nature* 353, 594; 17 October, 1991). *Mutsu* has spent most of the past 23 years in dock after developing a radiation leak on its maiden voyage. Conversion could cost as much as ¥20,000-million (\$150-million) and the finance ministry may well balk at funding two giant oceanographic vessels.

David Swinbanks

The West gropes for ways to help

Washington

SHORING up science in the former Soviet Union will be a long and expensive process that will require inventive new forms of international collaboration. But not doing so, say Soviet emigré scientists now living and working in the United States, could mean the loss of an entire generation of scientists in those newly freed countries.

What to do about this vast pool of talent was the subject of a special session at the annual April meeting of the American Physical Society last week. Like other scientific organizations, the APS is searching for ways to help the thousands of FSU scientists whose research budgets have been cut in half and who are facing further reductions as their governments try to stave off economic chaos.

A great deal is already happening. The National Science Foundation, for example, has recently offered an extra \$10,000 to 100 US scientists from various disciplines who are already receiving NSF support for collaborative projects with researchers in the former Soviet Union. The money can go toward anything from scientific equipment and office supplies to subscriptions and shipping. It does not cover salaries. If NSF had more money, says programme officer Gerson Sher, it would like to extend such supplemental awards to any recipient of an NSF grant with a worthwhile idea for joint research.

The Stanford Linear Accelerator Center is waiting for approval from the US Department of Energy for a \$10,000 subcon-

tract that will allow it to support some 20 theoretical physicists at the Physical Technical Institute in St Petersburg. The agreement is the latest to take advantage of the bargain-basement salaries that Russian scientists earn compared with their Western colleagues. In other cases, individual researchers have been remarkably successful after taking matters into their own hands (see accompanying story).

Everyone agrees that such time-honoured responses as travel fellowships and free copies of journals, although important, will not solve the problem. There is also consensus that the aid should by-pass the ex-Soviet bureaucracy and go directly into the hands of individuals. At its core, the issue comes down to the question asked during the APS meeting by Herman Feshbach of the Massachusetts Institute of Technology: "How do we identify the people we want to help?"

The answer, it seems, will require the research establishments in both the United States and the countries of the former Soviet Union to adopt new approaches to supporting talent. And there were plenty of suggestions of how to bring that about.

"Think of those groups with the most promising futures", said Edward Shuryak, a theoretical high-energy physicist at the State University of New York, Stony Brook, who arrived in the United States two years ago from Novosibirsk. "Ask them to put together proposals that US scientists can look at. Then give them small grants, and invite the best ones over for a better look."

At the same time, Shuryak predicted that the enormous difference in the exchange rate between the ruble and the dollar will not last forever, and he warned his colleagues not to start something that they cannot finish. "It will be more expensive in the long run to support scientists than it now appears", he said. "You must take that into account."

Efforts to aid such scientists must also find ways to deal with the large gap that exists between younger researchers and senior scientists who are already well-known in the West, according to Mark Strikman of Pennsylvania State University, formerly of St Petersburg. A successful programme, he believes, must involve both age groups.

"At Penn State we are trying to create small centers of four or five people," Strikman says. "It would be led by a senior scientist visiting from Russia, who would attract a rotating group of visitors. People would stay long enough to develop US contacts and then go back to Russia."

Such 'shuttle science', now being conducted at several universities, exposes scientists in the former Soviet Union to the Western approach to science funding, including a crash course on grant writing, peer review and other aspects of conducting science in a market economy. And, although not perfect, it works surprisingly well. As one former Soviet scientist now working in the United States pointed out, "You'd be surprised how quickly people learn on an empty stomach".

Jeffrey Mervis

US astronomers compute an answer

ASTROPHYSICIST Rashid Sunyaev is getting by with a little help from his friends. Sunyaev and his team of 600 scientists and technicians at the Institute for Space Research (IKI) in Moscow have worked for five years to prepare a massive X-ray and gamma-ray satellite for launch in 1995. It is an international effort, with researchers from France, Britain, the Netherlands and the United States providing half a dozen instruments that will be launched on a Proton rocket.

With two previous high-energy physics missions under its belt — one, *Kvant*, was launched serendipitously only a month before the explosion of the 1987A supernova and detected X-ray emissions from it — the IKI team has won the respect of astronomers around the world. But its ability to analyse data from *Kvant* and *GRANAT* (a gamma-ray telescope launched in 1989) missions, much less to handle the 1-gigabit-a-day output of the forthcoming satellite, has been limited by its available computing power, a handful of antiquated IBM-PC clones.

Enter Don Lamb, an astrophysicist at the University of Chicago and a long-time colleague of Sunyaev. Lamb is not a member of the team for the Spectrum X-Gamma mission, but when he learned in January of Sunyaev's dilemma he vowed to do something about it. "I told Rashid that one way or the other I would make it happen."

One thing he did was to call Bob Novick, a physicist at

Columbia University who is principal investigator on one of the instruments and a member of the mission's international scientific council, and Jerry Ostriker, an astrophysicist at Princeton with strong links to the Russian space science community. Within a few weeks, each of them had persuaded his university to contribute \$8,000 towards the purchase of modern workstations.

In the midst of his campaign, Lamb talked with Roald Sagdeev, the former director of IKI, who was visiting Chicago as part of the university's celebration of its 100th anniversary and who had been talking to officials from Sun Microsystems Company about ways they could help FSU scientists. Soon Lamb was talking on the telephone with Jordan Graham, European marketing manager for Sun, which had just opened an office in Moscow.

Sun more than doubled the university's contribution to the Russian space institute by donating nearly \$30,000 worth of equipment. The resulting package — three colour SPARC workstations with 19-inch colour monitors, 8 megabytes of memory and several other features, plus a CD ROM reader and a high-speed tape backup drive — was shipped to Moscow for a computer exhibition in early April. The IKI scientists, after being trained by Sun, simply took the equipment with them at the end of the show.

J.M.

Computing in the real world

Tokyo, Munich & London

OFFICIALS of Japan's Ministry of International Trade and Industry (MITI) have been touring the capitals of Europe trying to drum up international support for a massive government-industry project to develop next-generation parallel computers.

The Real World Computing project is MITI's follow-up to the once-feared but long-since-forgotten fifth-generation computer project. This time, rather than setting their sights on one particular type of parallel computer, MITI officials intend to take a multi-pronged approach looking at neural networks and optical computers, as well as massively parallel machines. And, unlike the fifth-generation project, which is primarily a domestic affair, MITI hopes to invest as much as 20 per cent of the proposed ¥100,000-million (\$750-million) budget for the ten-year project overseas.

Last month, MITI officials visited Brussels, Bonn, Paris and London inviting European researchers to join the programme. Earlier attempts to win the support of the United States were not very successful: the United States intends to participate only in a optoelectronic collaboration (see accompanying story), an area in which Japan is strong and has much to offer.

One idea being proposed by MITI is to establish a high-capacity computer link

between several laboratories in Europe and the new Real World Computing institute that will be set up in Tsukuba science city.

Although the reception of the MITI delegation in some countries was fairly cool, French and German researchers are quite enthusiastic about the Japanese project. Wolfgang Giloi of the German National Research Centre for Computer Science (GMD) in Berlin has offered to become a full partner in the project, and GMD has volunteered to host one of three remote laboratories connected by computer link to Japan that the Japanese wish to finance in Europe.

The French are more cautious. Laurent Kott, deputy director of the National Institute for Research in Computer Science and Automation, agrees that the project offers an important opportunity, but he says the exact nature of the scientific opportunities is "vague". Ian Watson of the University of Manchester notes that "the Japanese seemed to be [taken aback] by the fact that they got questioned by UK academics on the concrete goals of the project". Vagueness is characteristic of the early stages of Japanese projects, when a consensus on the direction is still being built. "It requires a certain leap of faith to join a Japanese national project" says the science officer of one European embassy in Tokyo.

But the Japanese are making efforts to

be more in line with the European approach, and Japan's laws have recently been re-written to allow participants in national projects to hold up to 50 per cent of patent rights. In the past, the government held all rights.

Nevertheless, some European researchers hesitate to join. Paul Refenes, a computer scientist at University College, London, says that Britain is likely to be "wary" of collaborating in areas where it is ahead of Japan, such as massively parallel computing. Collaborations in neural networking, where Europe and Japan are in equal states of advance, are more likely to be considered, he says.

The German and French ministries of research are trying to define their positions on the project at both a research and a political level, and France is lobbying for a common European position to be adopted.

In Japan, most of the major electronics companies, such as Hitachi, NEC, Toshiba and Fujitsu are expected to join. "Surprisingly", steel companies have also shown great interest in recent workshops on the project, says Yoshiki Mikami, director of MITI's Information, Computer and Communications Policy Office. A few Japanese subsidiaries of foreign companies, such as DEC of the United States, have also shown interest, but Mikami expects foreign participation to be confined "mainly to academics".

David Swinbanks, Alison Abbott & Ian Mundell

White House welcomes Japanese offer to help industry

THE US government is considering a proposal from Japan to give US researchers access to the Japanese industry's state-of-the-art optoelectronic components. Officials hope that a collaboration with Japan's Real World Computing project will unite the struggling US optoelectronics industry at a cost of just a few million dollars a year to the United States.

The arrangement, although favourable to US researchers, is not quite as one-sided as it sounds. Neither country will actually give up any proprietary technology. Instead, each country would build advanced components for the other, or swap specifications so that collaborating researchers can include previously unavailable components in their own optoelectronic devices.

As currently proposed, the collaboration would be based on the 'broker' principle, similar to that now being practised in a US/Japanese semiconductor collaboration known as MOSIS. In that model, a third party matches an inventory of potential users of state-of-the-art devices in one country with manufacturing facilities in the second country. In theory, no proprietary manufacturing techniques are needed transferred, but everyone gets access to the technology.

Under this system, a US researcher could obtain the specification of, for example, a laser available only in Japan. The researcher could then design a device around the specification, and either ship it to Japan to have the laser added, or request the part itself. Either way, the Japanese company need not share any of the proprietary details of how it makes the laser, or how it works.

Eugene Wong, associate director for physical sciences and engineering at the White House Office of Science and Technol-

ogy Policy, which is coordinating the US side of the negotiations, says that the method is a way to "use technology without acquiring technology" — just the right combination of what the US industry needs and what Japan is willing to give up. The US optoelectronics community is still fragmented, he says, and lacks a critical mass to sustain itself. The lure of Japanese technology could generate the type of collaboration between US industry and the academic community that is needed.

Before any agreement can be signed, however, the United States and Japan will have to resolve some philosophical differences. In the Japanese view, the goal of the collaboration is more joint research. That is because Japan, thanks in part to its camera industry, has a wide lead over the United States in optics technology. In keeping with their research emphasis, Japanese officials believe that the broker need not be an expert — in fact, the less the broker knows, the more willing the parties will be to cooperate without fear of exposing secrets.

Under the US model, however, brokers must understand the technology well enough to know what not to divulge to the other side. Wong suggests a non-competitive, yet informed, third party, such as a US university.

The two views may be reconciled during a six-month feasibility study involving half a dozen US agencies and industry groups, including the Optoelectronics Industry Development Association, a one-year-old group headed by Arpad Bergh of Bell Communications Research. Bergh expects little US opposition to the proposal. But it remains to be seen whether Japan wants US participation in its Real World project badly enough to accept the US view of a model collaboration.

CA & DS

Programme aids developing world

Washington

A US-BASED centre hopes to help developing countries take advantage of advances in agricultural biotechnology with the support of private companies and foundations that want to stimulate that technology.

Last month, the International Service for the Acquisition of Agri-biotech Applications (ISAAA) announced its home for the next five years would be at Cornell University in Ithaca, New York. ISAAA intends to help farmers increase yields and reduce their dependence on pesticides in a handful of countries that are best prepared to take advantage of the new technologies.

At present those farmers lack the money, technical skills and infrastructure to make use of this technology, which is becoming increasingly proprietary. What ISAAA hopes to do is tap into that extensive knowledge, now held mostly by private industry, and transfer it to developing countries without disrupting the way agriculture is carried out in those countries.

"That's the beauty of biotechnology", says Robert Fraley, who is director of plant science technology at Monsanto Company and a member of ISAAA's board of directors. At the same time biotechnology offers farmers the latest technology, he says, "you're providing it in a traditional seed package" that does not require any change in farming practices.

Previous efforts to transfer technology to developing countries have lacked sufficient people, technology and money in those countries. The models also have relied on laboratory-based initiatives between universities rather than people on the front lines. In contrast, ISAAA will invest not in laboratory facilities but rather in transferring tested technologies to those who can use them.

ISAAA has raised more than \$4 million in almost 10 months of operation, but David Altman, ISAAA's president and executive director, says it will need almost three times that amount to be fully operational. ISAAA also has found financial backers for two projects in Mexico and Taiwan.

The service plans to have branches around the world to help its staff learn about proprietary technology in those regions. In addition to its AmeriCenter at Cornell University, Altman hopes by next year to open a EuroCenter in Norwich, United Kingdom. Plans also are afoot for an AsiaCenter in Japan, as well as locating staff in Africa, Asia and Latin America.

ISAAA will concentrate on 10 target countries within Asia, Latin America and the Middle East/Africa — places that ISAAA feels have both the capability and political will to implement agricultural programmes in biotechnology, and where the chances for success are greatest. "We're not interested in creating a United Na-

tions, we're interested in moving the process forward", he explains. It will also confine itself to applications that have been tested in industrialized countries and where field testing in the developing country can begin within five years.

Initially, ISAAA will focus on plant biotechnology applications in the areas of tissue culture, diagnostics and transgenic plants. Altman says that he favours projects that help poorer farmers to grow non-commercial crops, in particular vegetatively propagated and open-pollinated fruit and vegetables. Other projects will focus on forestry and the use of micropropagation techniques to develop tropical tree species that will contribute to biodiversity and which are difficult to propagate through seed.

ISAAA has begun collaborative projects in Mexico and Taiwan and will soon begin a third with Costa Rica. Another 30 or so project proposals are in the pipeline. The Taiwanese project, with money from the US Agency for International Development, involves scientists from Washington State University and the Asian Vegetable Research and Development Center in Taiwan working together on a DNA probe to detect the presence of black rot in crucifers. The four-year Mexican project, which began last year with funding from the Rockefeller Foundation, hopes to extend proprietary virus-resistance technology, developed by Monsanto to protect North American potato varieties, to a locally grown Mexican variety of potato that accounts for 70 per cent of the national potato crop. It involves the Center for Advanced Research Studies (CINVESTAV) in Irapuato, Mexico, the Mexican Ministry of Agriculture and Water Resources and Monsanto.

The potato crop in Mexico, the country's fifth most important crop, suffers from serious losses due to viral diseases. Despite a \$4 million spray control programme, the reduction in annual yield can be as high as 25 per cent. Luis Herrera Estrella, head of genetic engineering at CINVESTAV, says that corn would have been a better target than potatoes because of its economic importance. But corn is also one of the most important commercial crops for agricultural companies in the United States. As a result, Estrella says, his centre had to pick a crop that was important to Mexico but one in which the transfer of technology "would not jeopardize the commercial interests of the company".

Robert Horsch of Monsanto says that the potato was chosen because the technology was available when the project was started two years. It was not possible at that time to genetically engineer corn, he says, and even today there are no products on the market in the United States.

Although Monsanto is commercializing the technology for North American potato varieties, it has waived its legal rights to the technology for use in the Mexican variety. "The potato project is an out-and-out donation of the technology", Horsch says. In the long run, however, Monsanto sees the project as an opportunity to build the contacts and infrastructure that some day will turn many of these countries into potential trading partners.

Mexican scientists being trained at Monsanto have already introduced the proprietary coat protein genes into the Mexican variety of potato, and Monsanto hopes to be able to conduct field tests of the virus-resistant potato in the United States by mid-summer. The scientists also are learning from Monsanto's experience with field-testing procedures and environmental pressure groups. In a related project, ISAAA is helping Mexican authorities to develop the infrastructure and regulatory biosafety procedures they need to test and introduce genetically engineered plant varieties.

Although good conventional plant breeding programmes also could improve crop yields in developing countries, biotechnology speeds up the process. At present it can take 12 years to produce a new wheat variety by conventional means, and seven or eight years for corn.

Diane Gershon

SEISMOLOGY

Quake news quickest

RESEARCHERS at the California Institute of Technology may not be able to predict the next earthquake, but once it happens they are usually the first to respond. Last week their experimental real-time earthquake information service passed a live test as Southern California was rocked by a earthquake measuring 6.0 on the Richter scale.

Caltech's Seismological Laboratory was able to broadcast seismological data on the quake within three to four minutes of the first shock. Data travelled from the university to a commercial radio pager network, then to receivers hooked up to computer terminals in the control rooms of its clients, five local utility companies and a railway company. The clients get information on the location, size, ground motion, amplitude and type of the earthquake, data they use to decide where to send repair trucks to look for damaged lines and service disruptions. Known as the Caltech-USGS [US Geological Survey] Broadcast of Earthquakes programme, the network has been in operation for about a year and a half and is the fastest in the country, according to its director, Hiroo Kanamori.

CA

Yangtze project dammed with faint praise

Hong Kong

THE credibility of Chinese scientists and engineers was called into question during a surprisingly public debate that preceded approval earlier this month of the long-planned Three Gorges Dam on the Yangtze River. Critics of the undertaking, said to be the world's largest hydroelectric project, have raised doubts about the ability of the country's technical community

Tony Waltham

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Plan to dam Qutang Gorge inflames critics

to carry out such a massive undertaking safely, at cost and without harming the environment.

Only two-thirds of the deputies attending this month's meeting of the National Peoples' Congress voted to approved the project. Although only 177 of the 2,600 deputies actually voted no, another 664 abstained. The vote itself was held in the midst of loud protests by those who said they had not been allowed to speak their minds.

The idea of building a dam across the Yangtze at Yichang, in the central Chinese province of Hubei, goes back some 40 years. The current version was approved by China's leadership in January.

The project has three goals: to prevent flooding of the Yangtze, to improve navigation on the river and to generate power for the surrounding communities. Flooding has been a periodic threat to residents; in 1954, for example, a flood killed 30,000 people and left one million homeless. In addition, a report submitted to the Peoples' Congress by the State Council's Three Gorges Committee declared that the project "is especially important as it can protect the livelihood of the 15 million inhabitants of the Jingjiang River area".

The 185-metre-high dam will have an annual output of 84,000 million kilowatt-hours. It will take 18 years to complete, at a cost of 57 billion yuan (\$US10,000 million). Just under half the cost will be paid by those who use the power it generates, beginning halfway through the project. The rest will come from bonds and from private sources.

Opponents have focused on several negative aspects of the project. In addition to inundating 19 towns and counties and 24,000 hectares of arable land, the project

will destroy a tourist region not unlike the Grand Canyon in the United States. The government says that nearly three-quarters of a million people will be forced to move from fertile farmland to much less desirable areas; critics say the figure could exceed a million.

Although international environmental organizations have been quick to criticize the project, the real surprise has been the extent of protest by deputies to the People's Congress, including the former vice-minister for water conservancy, Li Rui, and the former deputy director of state planning, Tian Fang.

The level of anger is especially high in Sichuan province, which is upstream of the dam. Provincial leader Zhang Haoru, who ultimately supported the project, nevertheless says that it will damage historical sites. Some scientists have suggested that a series of smaller dams could curb flooding and provide power without causing nearly as much damage to the environment as the Three Gorges project.

Critics have also told government officials that they expect to be kept informed of all potential problems. "We Sichuan

people, on the whole, support the dam", says Nie Rong-Gui, deputy secretary of the provincial communist party committee. "But problems such as reservoir siltation, mass resettlement and environmental impact have been exposed, and we hope that the central government will adopt serious measures to solve them so that we can all feel at ease."

Some of these issues were discussed in a report that was issued by the ministries of energy and water but withheld from deputies to the Peoples' Congress. One author, Wang Zen, a researcher at China's Forestry Society, spoke critically about the extensive logging that the project would require.

Government officials have not decided when to begin construction, but they expect the debate to continue. "In some year of the 1990s the project will be formally launched", says Yang Zhenhuai, the minister of water resources. "In the preparatory work and even during construction, all suggestions and opinions from different parties will be treated earnestly and respected."

Peter Gwynne,
with You Qin Li, Beijing

BIOTECHNOLOGY

Bad timing for BU's stock gamble

WALL Street has given a thumbs down to Boston University president John Silber's controversial decision to put nearly \$100 million of university money in a biotechnology startup.

Seragen Inc., the Hopkinton, Massachusetts, biotechnology company

that BU has built up with capital and loans, decided to go public after seeing some of the hugely successful stock sales of last year (see *Nature* 356, 183; 1992). But by the time the Seragen stock went on sale earlier this month, Wall Street had turned away from the industry (see accompanying chart) and the company was forced to reduce its asking price from \$17-\$20 per share to \$12-\$14. Instead of \$60 million, Seragen raised only \$36 million. And the price of the stock has continued to drop; last week it stood at \$9.50.

Those numbers are bad news for BU and the mercurial Silber, who has risked the equivalent of one-fifth of the

university's endowment in the company. Silber hoped that the public offering (the sale of three million shares represented a quarter of the company) would be successful enough to allow BU to stop spending \$1.2 million of its endowment each month to keep Seragen

running. The university still owns 69 per cent (8.3 million shares) of the company. But at \$9.50 a share, that stake is worth just \$78 million — \$20 million less than BU's investment.

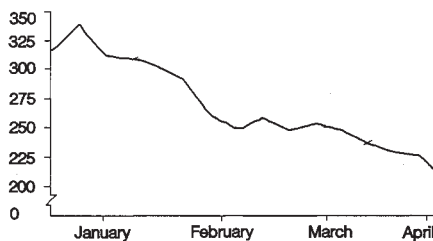
Despite the disappointing results, BU still plans to end its monthly subsidies to the company, prompted in part

by an order from the Massachusetts Attorney General to reduce the state-owned university's risk. That means that Seragen must find other sources for the capital it will need until its first products — fusion toxins for cancer and autoimmune disease therapies — appear on the market in another two or three years.

Christopher Anderson

Wall Street sours on biotechnology

Weekly stock index of 56 biotech companies
29 December 1989 = 100



Alex Brown & Sons, Inc.

UK panels need more clout

London

RESEARCH ethics committees (REC) in Britain do not have the muscle to carry out their job of overseeing clinical experiments on humans, according to a report issued last week* by the independent King's Fund Institute. But none of the several ways to make them stronger — including new legislation and clearer rules from their authorizing bodies — are likely to occur.

The ethics committees were set up somewhat haphazardly in the 1960s at the prompting of the Royal College of Physicians. There are about 270 committees, most answering to the district health authorities under which they operate. In addition to reviewing research on National Health Service patients, the ethics committees also consider volunteer studies at

universities and medical schools. A large proportion of the work involves therapeutic studies sponsored by the pharmaceutical industry, but the scope of activity goes from basic medical research to nursing studies.

Despite a willingness on the part of the medical research community and the pharmaceutical industry to accept some sort of legislative framework, it is unlikely that the UK government will go further than the general guidelines issued by the Department of Health last August. With the exception of the 1990 law that allows research on human embryos no older than 14 days, Britain has a distinct disinclination to legislate on medical ethics.

According to the author of the report, Julia Neuberger, a concerted effort by the

district health associations and other authorizing bodies could go a long way to solving some of the inconsistencies in the way committees are comprised and the way they operate. Britain lags behind other European countries, she says, citing legislation passed by France in 1990 that established consultative committees — similar in role to the RECs — in each region of France, with medical inspectors from the ministry of health overseeing the whole system. In the Irish Republic, pharmaceutical trials are policed by a team of inspectors from the National Drugs Advisory Board in Dublin, a system set up following the death of a healthy volunteer who was involved in more than one trial.

In addition, guidelines issued by the European Commission in 1990 on clinical trials are likely to be turned into a directive later this year. This already has the support of several European states, including Britain, but its effect is expected to be limited to preventing fraud.

The report found that some RECs were unsure whether to review the value of the research as well as the ethical issues it raised. Another worry was that clinical research is increasingly driven by the needs of the pharmaceutical industry, in particular 'me-too' drugs that are designed to challenge successful products.

The pharmaceutical industry does not think that the committees are the proper forum for discussing the value of proposed research. "That has nothing to do with ethics", commented Frank Wells, medical director of the Association of the British Pharmaceutical Industry. "Anything of likely therapeutic value should be tested."

But David Evered, second secretary to the Medical Research Council, says that the committees have an obligation to examine the issue of quality. "Any research which is unsound is unethical", he says. "There is a plurality of funding sources, some of which are softer than others, that may be used to support activities which have not been subject to peer review."

Evered agrees with the report's suggestion that RECs should have access to external expertise to assess the quality of research if their members are so lacking.

Ian Mundell

* Ethics and Health Care, King's Fund Institute, 1992; send £7.95 to Bournemouth English Book Centre, 9 Albion Close, Parkstone, Poole, Dorset, BH12 3LL.

SOUTH AFRICA

Beyond apartheid — on the cheap

Cape Town

In the wake of the dismantling of apartheid, South African scientists are busy renewing their ties with the rest of the world. They no longer seem to have problems obtaining visas for overseas visits, nor are they being excluded from international conferences. But reintegrating the country into the global scientific community seems likely to be slowed by the universal difficulty of obtaining funds for international initiatives.

In a recent example of shifting attitudes towards the formerly outcast nation, the American Physical Society (APS) has issued a statement encouraging interaction between individual scientists in the United States and South Africa. Referring to the financial and social stresses that have jeopardized the training of African scientists, the statement says that "the isolation of South African teachers and faculty from external colleagues makes it even more difficult to train and maintain a core of qualified scientists". But APS warns that, although the political climate at last favours closer ties to South Africa, available resources are likely to be applied to more pressing needs.

In the field of nuclear power, relations have improved rapidly since last summer, when South Africa agreed to abide by the Nuclear Non-Proliferation Treaty. The South African Atomic Energy Corporation (AEC) immediately invited cooperation from the eight member states of Afra, the African section of the International Atomic Energy Agency, under the aegis of the agency's technical assistance programme. Kenya and Ghana both agreed to visit South Africa.

The chief science secretary for the Kenyan National Council for Science and

Technology, Ammon Oyango, will nominate South Africa to become a member of Afra at an association meeting this week. South Africa and Kenya are due to draft a bilateral agreement on nuclear cooperation. According to Waldo Stumpf, chief executive of the AEC, such an agreement would allow South Africa to export radioactive isotopes for medical and industrial purposes to Kenya, and to train Kenyans in isotope production.

Stumpf says that South Africa's adoption of the terms of the non-proliferation treaty has generated a stream of visitors from leading nuclear powers. In addition, it has enabled the corporation to export uranium hexafluoride and enriched uranium for the first time.

South Africa remains excluded from two other international scientific organizations, the United Nations' Economic, Scientific and Cultural Organization (UNESCO) and its Environmental Programme. But it appears doubtful that the country would consider payment of UNESCO dues worthwhile and affordable at a time when science funding is shrinking (see *Nature* 356, 278; 1992).

Scientific cooperation must also conform to political developments. Ania Grobicki, national convener of the science and technology group for the African National Congress (ANC), says that it is premature for other governments to seek bilateral agreements with South Africa before a multiracial government is established there. Although the ANC encourages cooperation between individual scientists in South Africa and elsewhere, she says that "we are opposed to people seeking to sign agreements at a national level which it would be difficult for a future government to review". **Michael Cherry**

Corrections

A recent News item (*Nature* 356, 549; 16 April 1992) incorrectly stated that there are 20 PhDs in the genome centre at the Whitehead Institute of Biomedical Research in Cambridge, Massachusetts. The correct number is about 20 staff members in total, including students and technicians.

A recent News and Views (*Nature* 356, 473; 9 April 1992) mistakenly reported that there are 25,000 cases of tuberculosis in New York City. In fact, that number applies to the United States as a whole. The figure for New York City is 3,500 TB cases.

Problems of European law

SIR — Although 1992 should mark the establishment of a free market of individuals, goods and services in the countries of the European Communities and the European Free Trade Association (EFTA), there remain limitations on the movement of people (in this case a scientist) that should have been abandoned long ago. Instead, these limitations are enforced by recent laws.

I work at the National Cancer Institute in Milan, Italy. Having funds for a postdoctoral fellow, I advertised in *Nature* and received nine applications for this position, none of them from an Italian national. The most suitable candidate was a Chinese scientist (Z.Y.) who had been living in Switzerland since 1989 and who recently (February 1992) got his PhD in pharmacy from the University of Bern. I therefore invited him to Milan for an interview. At this point, the nightmare materialized. As he was Chinese, he needed a visa to enter Italy. A new Italian immigration law requires a minimum of three months valid residence permit in the country of residence before a visa can be issued. Z.Y. had only two months of validity (his visa in Switzerland was linked to his position as a PhD student) and was therefore not eligible for even a one-day visa. I called the Italian consulate in Bern, but nobody would take responsibility for granting the visa. My letter of invitation or my oath that Z.Y. would return to Switzerland after the interview made no difference. I was even offered friendly advice to hire an Italian national.

With an Italian sense of realism, I arranged to hold the interview in Chiasso (Switzerland), 40 km from Milan. I and another representative of my institution met Z.Y. in Chiasso and we decided that he was the right person for the position. However, my institution required that Z.Y. be physically present in Milan for at least one day before an official appointment as postdoctoral fellow could be made. But, because of the law, Z.Y. could not visit Milan, so he could not be appointed to the fellowship. Because he could not be appointed, he could not apply for a visa to reside in Italy. To make things even worse, Z.Y. was refused an extension of his residence permit in Switzerland.

The conclusion was that I lost the best candidate for this position; Z.Y. was forced to go back to China; and I wasted at least four days of my time in this fruitless attempt.

Although the general effect on illegal immigration of this new law is clear to everybody in Milan (at almost every traffic light there is an immigrant trying to wash one's windscreen), the law has

efficiently blocked the legal immigration of a skilled scientist who was needed for a position in Italy.

I have also come to realize that each EC and EFTA country has its own different immigration law and that Z.Y., for example, could have gone to France with little difficulty, although he could not enter Italy. This situation is not only unproductive and unacceptable, but also underlines the huge difficulties that still lie (nine months from the unitary market of 1993) between us and a truly United Europe. For some people, the trip from Bern to Milan (about 300 km) is still full of hidden dangers in 1992.

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Productivity drive

SIR — In a recent study on prolific productivity among prominent scientists (*Medical Hypotheses*, in the press), I have identified eight scientists who have been authors of more than 1,000 research publications, including books, monographs and patents as well as regular papers. They are, in chronological order, Thomas Alva Edison, Paul Karrer, Margaret Mead, Giulio Natta, Hans Selye, Herbert C. Brown, Tetsuji Kametani and Carl Djerassi. Among these, Karrer, Natta and Brown were Nobel prizewinners in chemistry. Four criteria that I was able to identify as clues to their prolific productivity are (1) enthusiasm for compulsive work and eccentric life style, (2) physical and/or environmental handicap, (3) pioneering efforts in a new research field and (4) selection of research area, predominantly organic chemistry. If one takes into account the publications of scientists on social and political issues as well, I believe that Bertrand Russell and Linus Pauling (both Nobel prizewinners) also share the credit for prolific productivity, lasting for seven decades.

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'Neutral' CFCs?

SIR — The suggestion¹ that CFCs may be 'greenhouse neutral' on a global scale may bear on the negotiations at Rio de Janeiro in June aimed at reducing the potential for global warming.

Although spatial variations of ozone concentration will complicate the argument², at first sight the possibility of neutrality diminishes the credibility of the Bush Administration's position that CFC emissions should be traded off

against carbon dioxide emissions in a comprehensive approach³. In any case, this development indicates the difficulties that might arise as a result of changing knowledge if such an approach was adopted⁴, and should serve as a warning that the question of carbon dioxide reductions cannot be avoided.

The result also challenges the administration's view that global change research has 'matured' and requires less funding for basic research⁵.

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Gene patents

SIR — Craig Venter and colleagues recently expanded their original list of expressed sequence tags (ESTs)¹ to include an additional 2,375 sequences from human brain cDNA libraries². The appropriateness of obtaining patents on these sequences has generated well publicized controversy. Not so readily publicized is that in at least eight cases the Venter group appears to be applying for patents on sequences from the Bluescript sequencing vectors. I believe that Stratagene³, the owner of these popular vectors, may be likely to object to the award of patents to the Venter group for these sequences.

A search of the Genbank database⁴ that I conducted shows the following ESTs to contain Bluescript polylinker sequences: Est02006, Est01040, Est01016, Est01479, Est00328, Est02460. In addition, the following ESTs contain vector sequences which flank the polylinker: Est01431 and Est01430. The fact that these entries contain vector sequences may be indicative of the degree of analysis to which they have been subjected. Furthermore, it may shed light on the general advisability of obtaining patents on the entire set of EST sequences without further analysis of the individual sequences.

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The growing inaccessibility of science

Donald P. Hayes

That science has become more difficult for nonspecialists to understand is a truth universally acknowledged. Here is a measure of the extent of the process.

THERE is plenty of anecdotal evidence that large areas of the scientific literature are becoming incomprehensible to all but a few initiates. But how persuasive is anecdote? In this article I describe an objective way of looking at the matter and discuss its application to science journals over the past 145 years. The approach is a method for measuring text difficulty. The data are taken from articles describing research in four categories of publication: general science (*Nature*, *Science* and *Scientific American*); ten professional journals in astronomy, biology, chemistry, geology and physics; science textbooks for introductory college courses; and popular science magazines.

In a nutshell, the analyses confirm impressions that research papers are written for specialists. This style means that authors can be explicit in their referencing and economical with space. But whereas the approach produces succinct papers for editors and referees, it makes tough reading for nonspecialists.

In measuring the difficulty of a piece of writing each sample text is assigned a difficulty scale score based on its choice of words from the full English lexicon (see box). The higher the score the more difficult the text. The table indicates the scale's use, range and validity, and Fig. 1 shows the results of analysis of research articles in *Nature* and *Science*, and of articles in *Scientific American*, published between 1930 and 1990. *Scientific American* does not publish reports of original research, whereas the other two do.

For 125 years, between 1845 and 1970, the use of vocabulary in *Scientific American* was at or slightly below the level of a modern newspaper (0.0); indeed, *Scientific American*, for its first 75 years, was a weekly newspaper of technology and science. Its language began to resemble that used in professional science journals after 1970. Interestingly, when the difficulty of the average article approached 15, there was a decline of over 125,000 subscribers, implying that many readers found texts written at those levels too opaque. When the level of *Scientific American* later dropped towards 10, there was a coincident increase in subscribers.

During *Nature*'s first 78 years (1869 to 1947) it was not necessary to be trained in science to read its contents because they were written near the 0.0 level.

RANGE OF LEXICAL DIFFICULTY IN SELECTED TEXT CATEGORIES

<i>Nature</i> (article on the transhydrogenase reaction, 1960)	55.5
<i>Science</i> (abstracts of Report articles, 1990)	44.8
<i>Cell</i> (articles, 1990)	38.0
<i>Nature</i> (research articles, 1990)	31.6
<i>Science</i> (research articles, 1990)	28.0
<i>Physics Today</i> (articles, 1990)	13.3
<i>New Scientist</i> (articles, 1986)	4.0
This manuscript	2.6
International English-language newspapers (N=30)	0.0
<i>Discover</i> (popularized science, 1990)	-4.7
Adult books, fiction, American	-19.3
<i>Ranger Rick</i> (natural history magazine for children)	-22.6
Comic books, British and American	-26.8
Children's books, fiction, British, age 10-14	-27.4
Children's books, fiction, American, age 9-12	-32.3
Adult to adult conversations, casual	-41.1
Mothers talking to their 3½-year-old children	-48.3
Farm workers talking to dairy cows	-59.1

(Data from the Cornell University Corpus³.)

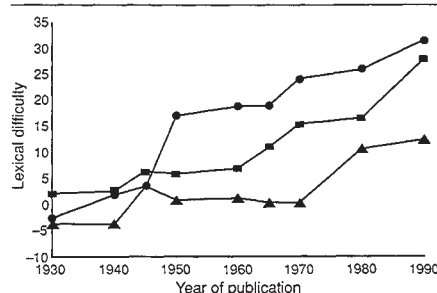


FIG. 1 The rise in lexical difficulty in *Nature* (●), *Science* (■) and *Scientific American* (▲) between 1930 and 1990.

Nature became the first general science journal to show a change, and since 1947 its research articles have become harder to read in each successive decade. *Science* began, in 1883, at -8.5. In its first 77 years, the main articles remained at or slightly above newspaper levels. A change in the text difficulty in *Science* did not emerge until 1960, but since then its articles too have grown much more difficult.

Although the impetus for this trend lies with research discoveries and theoretical developments, from the abruptness with which the changes in text difficulty

occurred in all three publications it seems that editorial policy may have had something to do with it. Editorial policy affects how major articles and short reports are selected; how and for whom papers are written; and which fields in science are to be featured. One way in which the level of difficulty in *Nature* and *Science* changed was that fewer natural history papers were published (these are often descriptive and generally written at lower levels of difficulty), natural science papers (which are more analytical, and usually written at higher levels) being substituted instead.

What of the basic science journals? There too the trend is clear (Fig. 2). All ten of the journals analysed grew more difficult, and each was growing more difficult in every period between 1900 (or its founding) and 1990. There are few signs that the process is slowing.

The rates at which these journals changed and their most recent levels of difficulty, however, vary. For instance astronomy and physics journals are written at lower levels than those in biology, chemistry and geology. But because physics and related fields make the heaviest use of equations, their lower difficulty could well be an artefact — lexicographers do not consider equations to be words, and so exclude them from dictionaries and lexical analyses. Articles in biology, chemistry and geology, by contrast rely heavily on their exceptionally large technical lexicons to describe their complex and highly differentiated subject matter.

Coincidentally or not, major college textbooks for introductory physics (0.1) and astronomy (-6.5) were also written at lower levels than those for biology (4.5), chemistry (5.6) and geology (11.1). Equations were rare in all of those texts. Aside from the contribution formalizations make to text difficulty, every physics-related journal grew in lexical difficulty between 1950 and 1990: *Astrophysical Journal* rose from 3 to 18; *Icarus*, 10 to 21; *Physical Review A*, 6 to 17; *Physical Review D*, 10 to 15; and *Journal of Geophysical Research*, 7 to 16.

There are no doubt several contributory factors to science, as written, becoming tougher to understand. One of course is that scientific understanding has become ever more detailed. Another is the dynamics of publishing. Like fish

Text analysis

ONE of the main contributors to a text's difficulty is its pattern of word choice. In English, this choice is from an estimated 600,000 word-types (terms having unique orthography). A log-normal model¹ of word choice predicts that when the words from a large representative sample of texts are arrayed by the log of their frequency of use, the resulting cumulative distribution will be linear. British and US newspapers have closely followed this pattern of word choice since at least 1730. Because of this stability, the pattern's simplicity and their wide readership, newspapers were adopted as the standard for comparison.

Taking social interactions into account, however, leads to a more complex and general model. Speakers and authors normally tailor texts to their intended audience's interests and knowledge. Compared to newspaper word choice, texts of spontaneous speech underuse the more common grammatical words, overuse the more common substantive words and underuse the rarer substantive words, producing an S-shaped cumulative distribution. Difficult technical texts have the opposite biases, producing the reverse S-shaped distribution. Lexical difficulty is represented by this spectrum of lexical patterns.

The software used in the work described here calculates the discrepancy between a specific text's pattern of word choice and the linear pattern of newspapers. First, each text of 1,500+ words is derived by multi-stage stratified simple random sampling and edited to a common standard. Second, a cumulative curve is generated from the words in that text beginning with the proportion of the most common English word, 'the'; to which is added the proportion of the second ('of'); the third ('and'); and so on through the 10,000th most common word. (Reliable estimates for word frequencies beyond 10,000 are not available.) Third, the 75 most common words in English, accounting for about half the words in texts, are deleted as they contain little information.

Finally, the area beneath that text's cumulative curve is integrated and subtracted from the corresponding area beneath the cumulative curve for newspapers. Texts with negative lexical difficulty scores are skewed towards common words; those with positive scores are skewed towards rare words. The values quoted in this article represent the extent to which word choice is skewed relative to that of newspapers.

D.P.H.

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on a reef, science magazines must compete for essential resources: important authors and papers, subscribers and, for some, advertisers. They may have to compete for or exploit lexical niches as well.

For example, in the late 1970s it must have become apparent to other publishers that *Scientific American* had left its old niche at 0.0 and was not going to return. In the United States, four general science magazines were created to fill the gap. *Science Digest* transformed itself from a *Readers' Digest* format into a *Scientific American* look-alike (-2.6 in 1986). The American Chemical Society changed the name of the publication *Chemistry* to *SciQuest*, and broadened its message, coverage and appeal (2.2 in 1986). The American Association for the Advancement of Science (publisher of *Science*) developed *Science-80* (-1.0 in 1986) to fill a void in part created when the research articles in *Science* had risen in difficulty from 7 in 1960 to 17 in 1980. Only *Discover* survives (-0.4 in 1986, but -3.6 in 1992). For a brief period, all four magazines occupied the 0.0 niche.

The growth of science has greatly enlarged the audience for general and technical science publications. As their technical articles became more difficult, the general science journals and magazines vacated their former lexical niches. These were soon filled (coincidentally at the vacated levels) by new publications or by ones which moved there from some other niche. Such publications now fill most niches between -22.6 and 38. In particular, professional societies and science publishers have produced several single-science magazines tailored to specific audiences (for example *Physics Today* 13.3, *BioScience* 16.8, *Geology Today* 11.2, and *Chemistry in Britain* 12.6). There is even a chemistry newspaper, *Reaction Times* (7.8). A final adaptation to this trend has been for journals to differentiate parts of each issue, setting each section to a different lexical level, so all readers will find something they can read.

What, though, are the consequences of the drift towards inaccessibility? Specialization in science has produced unprecedented levels of knowledge, but the unwelcome side-effects are clear. These days, more expertise than ever is required to understand published research and theory in other fields and to referee papers and proposals in one's own

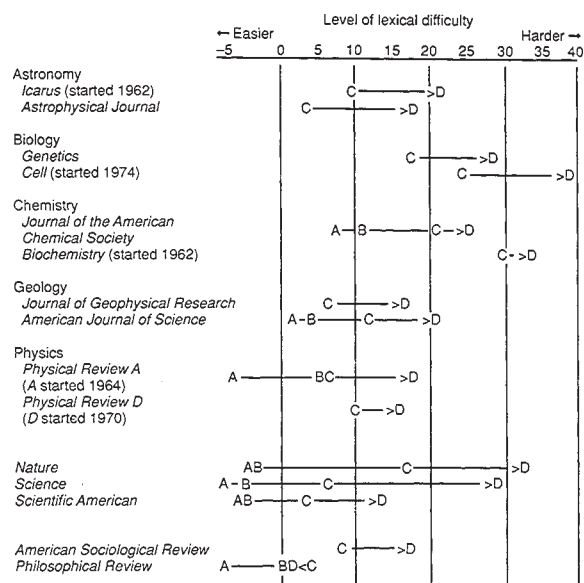


FIG. 2 Change in lexical difficulty in ten basic science journals, the three general journals and two journals dealing with other disciplines. A, 1900; B, 1925; C, 1950 or the first year of publication; D, 1990.

discipline. The broad consequences are that ideas flow less freely across and within the sciences, and the public's access to (and maybe trust in) science is diminished.

To scientists this trend represents a narrowing of their range of expertise, even while the depth of their knowledge grows. So they may change specialities less often as the costs of becoming expert in another area grow. One response, I suspect, has been an increase in collaboration with scientists in other specialities. Another has been to develop still more complex research teams whose members have complementary skills and knowledge. Complicated sociological structures such as this can be productive but they introduce new kinds of tension, for instance disputes over the order in which names appear on a paper.

Projecting the trend summarized in Fig. 2, there will soon be basic science journals whose average article difficulty will exceed 40, and before long some journal may consistently exceed 50 (indeed, many articles in *Cell* currently exceed 40, and a few now exceed 50). No mainstream science journal was as high as 10 in 1900. And of the nine journals examined and published as recently as 1950, only one was above 20. This erection of higher and higher barriers to the comprehension of scientific affairs must surely diminish science itself. Above all, it is a threat to an essential characteristic of the endeavour — its openness to outside examination and appraisal.

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Cosmology back to the beginning

Faint ripples in the cosmological microwave background, revealed by NASA's COBE satellite, were the seeds in the Big Bang that eventually became large-scale structures in the Universe.

TEMPERATURE variations in the 3-K cosmological background, imprinted some 10^{-35} seconds after the initial Big Bang singularity, at the era of inflation, have at last been found, 27 years after the microwave background itself was discovered by Penzias and Wilson. At the American Physical Society meeting in Washington DC last week, the COBE (Cosmic Background Explorer) team reported detection of the long-sought anisotropies, at a level of only $15 \mu\text{K}$. The angular scale of the fluctuations is so large (from 10° to 90° on the sky) that no causal process in standard non-inflationary cosmology could have generated them. We are viewing the birth of the Universe.

All structure in the Universe, according to Big Bang gospel, evolved from infinitesimal density fluctuations. Gravitational instability caused their inexorable growth and eventual development into galaxies, clusters of galaxies, and even larger structures. We observe today structure on scales of up to 100 megaparsecs (Mpc) in the nearby Universe. Yet its origin from density fluctuations has proved difficult to confirm. The reason is simple: such fluctuations were the only

deviations from homogeneity in the early Universe.

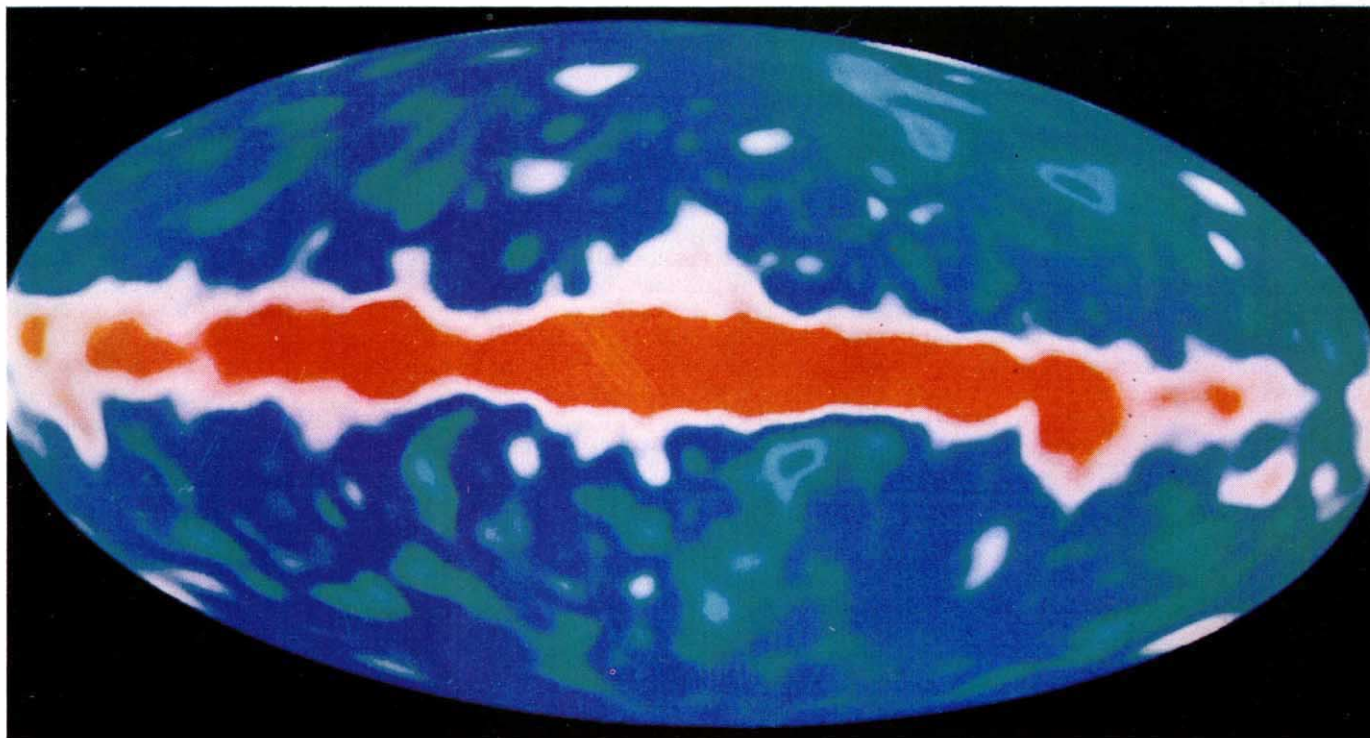
About a million years after the Big Bang, the density of matter dropped and the radiation temperature cooled to the point at which atomic hydrogen became the predominant state of matter. Before that, the Universe was an ionized plasma in which radiation scattered frequently off electrons, and the two were therefore in intimate thermal contact. But once atomic matter was prevalent, the scattering ceased and radiation was no longer entrained by matter. Travelling freely from then on, but cooled by cosmic expansion, these photons are now directly observable as the cosmic microwave radiation, which is therefore a pale relic of the fireball that dominated the Universe for the first 10,000 years.

We have two reasons to be reasonably confident in our knowledge of the Big Bang between a few milliseconds and a few years of cosmic time. Synthesis of light elements from hydrogen, particularly helium but also deuterium and lithium, left a remarkably robust legacy of the first few minutes. The Planck spectrum of the cosmic fireball was

bequeathed to us after the first few months of cosmic history, when thermalization first became inefficient. The COBE satellite and a rocket experiment have already reported that the cosmic microwave background is a true black-body at 2.736 K, with deviations no greater than a quarter of a per cent.

These results are the cornerstone of the hot Big Bang cosmology, providing persuasive evidence that the Universe was at one time hot and dense. But a perfectly uniform Big Bang would be unacceptable: there must have been deviations early on to make, over the subsequent billions of years, the obvious structure of the modern Universe. Temperature fluctuations in the microwave background measure the amplitude of these cosmological density fluctuations near the beginning of the era of matter domination, when this process started in earnest.

Until last week's announcement from the COBE team, there were only upper limits to microwave background fluctuations on various angular scales. These limits were enough to rule out models in which galaxies were made solely from baryonic matter, and constituted a strong



Microwave map of the Universe from COBE. The colours represent fluctuations of just $0.27 \mu\text{K}$ (warmest, orange).

rationale for cold dark matter cosmologies. Cold dark matter, weakly interacting particles that are hypothetical relics of the Big Bang, interacts only gravitationally with conventional baryons and radiation, so fluctuations in its distribution can start growing while baryonic matter is still trapped by radiation. When baryons eventually break loose, they fall into the potential wells created by cold dark matter, allowing large-scale structure to arise from much smaller initial baryonic matter fluctuations, with correspondingly smaller variations in the microwave background.

At the transitional epoch when matter and radiation go their separate ways, the horizon scale (how far light travelled since the Big Bang) is naturally imprinted on density fluctuations, and shows up in the distribution of matter today. This scale, about 10 Mpc, is the only natural scale in the cold dark matter spectrum of density fluctuations. Because galaxy clustering is relatively strong on scales of a few megaparsecs, one can normalize theory to observation on a scale where the physics is thought to be simple, and predict, once one has an idea of the initial fluctuation spectrum, what one might expect on much larger scales.

The theoretical breakthrough that came with inflationary cosmology, proposed by Guth in 1980, led to a specific prediction of the shape of the fluctuation spectrum as a function of scale. Inflation, through the presence of a vacuum energy associated with a particle-physics phase transition, causes a period of exponential cosmological expansion; it also blows up tiny quantum fluctuations in spatial curvature to scales that are macroscopic today. The most compelling versions predict equal curvature fluctuations on all scales. This is the famous ' $n=1$ ' spectrum, previously advocated on grounds of simplicity by Harrison and Zel'dovich. This spectrum is not unique to inflation, but what is remarkable is that inflation predicts fluctuations of this form on scales so large that there has been no subsequent causal contact that could have either generated or destroyed them.

This was the situation on the morning of 23 April 1992. Fluctuations in the fireball radiation, predicted¹⁻³ by theorists since 1967, had been refined^{4,5} to remain just below available experimental upper limits. Their primary cause was gravitational potential fluctuations at last scattering of the radiation, and the scale-invariant spectrum led to the prediction^{6,7} of temperature fluctuations of about 1 part in 10^5 over angular scales from 1° to 90° . Above 10° , the prediction refers to acausal fluctuations coming to us directly from inflation; near 1° , one could make a more direct connection to the precursors to structure on 100-Mpc scales. Minimal fluctuations were predicted, on the basis

of faith in a gravitational instability origin for galaxy clustering, superclustering and large-scale flows^{8,9}.

At the Washington meeting, several groups reported results of their new searches for temperature anisotropies. Observations from the South Pole (University of California, Santa Barbara) and with balloon-borne telescopes (Massachusetts Institute of Technology; University of California, Berkeley) revealed fluctuations at a level of between 1 and 3 parts in 10^5 over angular scales



Arno Penzias (left) and Robert Wilson with their microwave antenna that started the cosmic background exploration 27 years ago.

between 0.5° and 10° . But emission from our Galaxy was seen in the form of low-level fluctuations in diffuse interstellar synchrotron emission, bremsstrahlung and dust emission, and whether any of the temperature variations constituted a residual signal due to the cosmic background remained unresolved.

The Differential Microwave Radiometer (DMR) on board COBE, as reported by G. Smoot (University of California, Berkeley), has important advantages over the rival experiments. Intensity measurements were made at three frequencies, astutely chosen to be both near the galactic minimum and the cosmic maximum intensity (31.5, 53 and 90 GHz), allowing cosmological and galactic emission to be distinguished by their different spectral forms.

The DMR has two receiving horns, each equipped with two separate detectors for all three frequencies, pointing 60° apart on the sky, and noise reduction down to a few parts per million is achieved by comparing all the different outputs. The DMR maps the full sky every six months, and with one full year of data now analysed, the experimenters have unambiguously measured anisotropies in the microwave background. The fluctuations are consistent with the expected blackbody spectrum and have a characteristic amplitude $30 \pm 5 \mu\text{K}$ over 10° – 90° . About a tenth of this is due to emission from high latitudes in our Galaxy. This leaves a true cosmological

quadrupole signal with amplitude $16 \pm 4 \mu\text{K}$, or $\Delta T/T = 5(\pm 1.5) \times 10^{-6}$. This is more than a hundred times smaller than the dipole anisotropy due to our motion relative to the cosmic microwave background. The remaining cosmic fluctuations on scales from 7° to 90° have an amplitude of about 6×10^{-6} (in terms of the amplitude of the angular correlation function), and yield an index $n = 1.1 \pm 0.6$, consistent with the scale-invariant prediction of inflationary cosmology.

The observed amplitude indeed matches the density fluctuations required to account for large-scale structure, suitably extrapolated to larger scales according to the scale-invariant prescription. Cold dark matter proponents had even predicted the amplitude, provided one takes the minimal cold dark matter model in a Universe of critical density, without resorting to biasing (peaking) the distribution of luminous matter relative to dark matter. There is a flaw here, worthy of attention: such a model is a disaster on scales of a few megaparsecs, where it predicts excessive gravitational power. Correct the small-scale problem, and cold dark matter fails on large scales.

Other models lurking in the wings bypass inflation entirely, having density less than the critical value, or relying on topological defects such as strings, textures or late-forming domain walls to seed large-scale structure. They also give a quadrupole and low-order multipole structure in the microwave background that is distressingly similar to the scale-invariant, inflationary prediction.

So far, there is no self-consistent set of initial conditions that matches the observed Universe both on large and small scales. Perhaps we should only trust the very large scales where density fluctuations are still small, the nonlinear gravity of galaxy and cluster formation being a complex phenomenon that is still not fully understood. Nevertheless, the detection of fluctuations at more or less the predicted level is a remarkable achievement.

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Guides to experiments

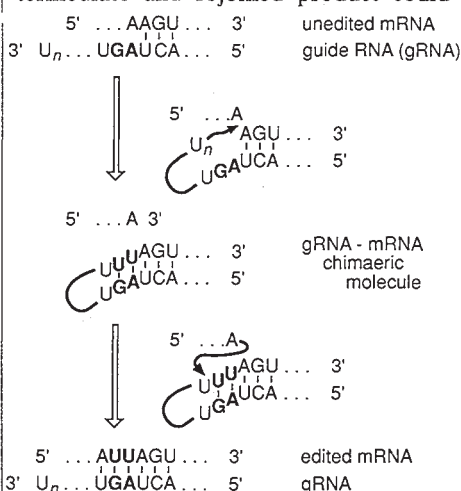
Barbara Sollner-Webb

RNA EDITING is a bizarre and fascinating form of gene transcript maturation, in which uridine residues are post-transcriptionally inserted into (and occasionally deleted from) mitochondrial RNAs in trypanosomes and related organisms to achieve the mature messenger RNA sequence. The process accounts for over half of the residues in certain mRNAs, but investigations into its mechanism have been hampered by the lack of a system that can be experimentally manipulated. Now, two reports — one in *Cell*¹ and one on page 807 of this issue² — describe *in vitro* systems that convert pre-mRNA into what is a widely regarded as an intermediate in the editing process. This advance promises to open the field of trypanosome RNA editing to direct experimental manipulation.

Since the discovery of trypanosome RNA editing in 1986³, and its wide recognition two years later following the demonstration that these mitochondrial transcripts can be massively edited⁴, much has been deduced about editing by analysing cellular RNA and DNA. This type of editing was found to progress in a 3' to 5' direction⁵, to generate partially edited molecules of unexpected heterogeneity⁶, and to be developmentally regulated⁷. At first, the fundamental issue was where the information for RNA editing is encoded. This question was answered with the discovery of 'guide' RNAs (gRNAs)⁸, short mitochondrial transcripts that are complementary to segments of edited mRNA (allowing for G:U base pairing). They are therefore likely to 'guide' the U additions and deletions of editing. But how do they function?

For a number of years it had been believed that editing involves cycles of mRNA cleavage, U addition (or deletion) and religation; the corresponding gRNAs could direct this process (see, for example, ref. 8). Then, last year, editing was proposed to occur instead by sets of concerted transesterification reactions^{9,10}, an idea which is based on the Nobel prize-winning transesterification mechanism for intron removal (see ref. 11). In the transesterification model, the oligo-U tail that is present at the 3' end of the gRNA would first attack the mRNA within the editing domain (the reaction indicated by the upper vertical arrow in the figure). This would form a chimaeric intermediate in which the gRNA is covalently joined to the 3' portion of the mRNA¹⁰. In a second transesterification reaction, the 3' end of the released mRNA fragment would

attack within the oligo-U track of the chimaeric molecule, liberating the gRNA and rejoining the now partially edited mRNA (reaction indicated by the lower vertical arrow in the figure). Indeed, such gRNA-mRNA chimaeric molecules have been identified in trypanosome mitochondrial RNA¹⁰, and this finding has provided support for the transesterification model. However, the same gRNA-mRNA chimaeric intermediate and rejoining product could



Scheme for RNA editing involving concerted cleavage-ligation reactions. The unedited primary transcript (top line) becomes edited at a site under the direction of a guide RNA (gRNA, second line); gRNA residues (shown in bold) 'guide' insertion and deletion of U residues (also shown in bold) in the mRNA. A gRNA-mRNA chimaeric molecule (middle diagram) is almost certainly a reaction intermediate; its formation *in vitro* is reported in the current papers^{1,2}. In the transesterification model^{9,10}, each cycle of editing involves two sets of concerted, energy-conserving cleavage-ligation reactions.

also be formed by separate cleavage and ligation events¹². To assess these and other proposed editing models experimentally, a faithful *in vitro* editing system is clearly needed.

Harris and Hajduk¹ and Koslowski *et al.*² now report a highly promising advance: the formation of gRNA-mRNA chimaeric molecules *in vitro*. Both groups used trypanosome mitochondrial extracts and exogenously added mRNA and gRNA. In Harris and Hajduk's studies on the transcript of the cytochrome *b* gene, the gRNA is joined through its oligo-U tail to the 3' portion of the mRNA. The junction is either at the most 3' (and therefore possibly the first) editing site, or it may be at the adjacent position (where an endonuclease of the extract cleaves this RNA¹³). Thus these chimaeras are the same as or

extremely similar to those expected to arise *in vivo*. In the studies from Stuart's laboratory² on the transcript of the ATPase 6 gene, the chimaeric molecules exhibit heterogeneous junctions, with numerous sites within the mRNA editing domain joined to a variety of positions in the 3' end of the gRNA. Although these chimaeras almost invariably lack the U residues from the original 3' end of the gRNA, they are otherwise very reminiscent of the chimaeras formed *in vivo*. Both of the *in vitro* systems^{1,2} show substrate specificity, because other control RNAs do not participate in chimaera formation.

It will, of course, be important to demonstrate that the chimaeras formed *in vitro* are generated by the same mechanism as that operating in the trypanosome. For instance, trypanosome mitochondrial extracts contain RNA ligase¹⁴ as well as the editing region-selective endonuclease noted above¹³, and these activities could generate chimaeras *in vitro*, irrespective of whether transesterification is the editing mechanism *in vivo*. In fact, a similarly specific formation of cytochrome *b* gRNA-mRNA chimaeras *in vitro* has been observed by Ken Piller in my laboratory using a heterologous endonuclease and RNA ligase, in the absence of any trypanosome factors.

The availability of *in vitro* systems for gRNA-mRNA chimaera formation should open the door to the direct experimental investigation of the editing mechanism. The new reports^{1,2} demonstrate the need for a 3'OH on the gRNA, which is consistent with the transesterification model of editing^{9,10}. But it is equally consistent with chimaera production being catalysed by endonuclease and RNA ligase. Further experiments will certainly resolve this issue. The next milestone in the development of an *in vitro* system will surely be to reproduce an entire cycle of editing (rejoining the mRNA backbone with appropriately inserted or deleted U residues), the goal being faithfully to reproduce the com-

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plete editing process *in vitro*. Encouraging preliminary evidence is now being observed in a number of laboratories.

One outstanding matter likely to be resolved is how the gRNAs guide the editing. Do they precisely dictate each editing modification or do they selectively preserve sites that become correctly edited in a less rigidly directed⁶ cleavage/joining system? An understanding of the basis of the developmental regulation of editing should also emerge. And this

knowledge may well provide insight into the intriguing evolutionary issues implicit in RNA editing. We can be optimistic that a popular topic for journal clubs a year hence will be the great progress in understanding the mechanism of trypanosome RNA editing that derives from these *in vitro* systems. □

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CLIMATE

Deep ocean circulation puzzle

Rainer Zahn

NOTIONS of the influence deep ocean circulation can have on the climate will have to be revised in the light of papers on pages 757 and 783 of this issue. Lehman and Keigwin¹ and Veum *et al.*² have examined tracers of ocean circulation in the northern corner of the North Atlantic and find that changes there 10,000 years ago, when the Earth was emerging from the last Ice Age, were not responsible for sudden, temporary regressions to glacial climates, as has been suggested.

If extremes of behaviour in a system are a good indicator of its underlying processes, then the Younger Dryas cold episode, a brief glacial snap 10,000 years ago during the global warming that brought our planet out of the last Ice Age, is an outstanding clue to the climate's inner workings and an outstanding example of the internal discontinuities it is susceptible to. Although external forcing such as solar heating increased gradually towards warmer conditions, the associated climatic transition was far from running smoothly. Several climatic anomalies are documented for this transition, the most severe being the Younger Dryas (named after *Dryas octopetala*, a flower typical of polar climates). Impressed by its apparent abruptness and severity, oceanographers and climatologists sense that this sharp climatic anomaly, which lasted only a few centuries, may reveal much about the operation of ocean-climate processes. The emergence of high-resolution accelerator techniques for dating sediments and microfossils in the mid-1980s provided an essential pre-requisite for studying such abrupt climate change and triggered a bloom of research on the Younger Dryas (see the accompanying chronology).

At the heart of the problem lies the way the North Atlantic Ocean circulated during various phases of the last glacial-interglacial transition. In the modern ocean, flow of warm saline surface waters from the tropical and subtropical

Atlantic to the high-latitude North Atlantic, accompanied by evaporation (and so cooling and increased salinity), is apparently required to trigger sinking of dense polar surface waters and formation of North Atlantic deep water. Advection of surface waters from the south, subsequent sinking and horizontal escape at depth may be viewed as a self-sustaining circulation loop which is part of the larger-scale water mass exchange between the North Atlantic and the rest of the world's oceans. The driving force which keeps this so-called "conveyor belt circulation"³ in motion is the flux of density due to the transport of salt through the North Atlantic^{4,5}. Modulations of the conveyor belt due to varying inputs of meltwater from disintegrating ice sheets have been called upon to explain the Younger Dryas climatic deterioration during the global warming of the last deglaciation³. The injection of meltwaters would overwhelm freshwater loss from surface waters by evaporation and lower the salinity of North Atlantic surface waters to an extent that the density contrast between surface waters and underlying deep waters becomes too large to be compensated by winter-time cooling at the surface. Water convection would cease and the conveyor belt would become drowned out of existence. Because warm saline tropical waters would stop flowing into the subarctic North Atlantic, climate in the region would deteriorate to almost fully glacial conditions.

Meltwater discharge into the North Atlantic did not continue evenly over the last glacial-interglacial transition (between 14,000 and 8,000 years ago). There were two meltwater pulses, separated by about 2,500 years, from distinct episodes of disintegration of the North American Laurentide Ice Sheet⁶. The Younger Dryas cold episode started just after the first meltwater pulse and ended shortly before the second one: instead of happening alongside elevated meltwater input, the Younger Dryas is centred

right in the low between the two pulses, when melting came close to a halt.

This is exactly what Lehman and Keigwin¹ observe by looking at microfaunal and stable-isotope distribution patterns along a highest-quality sediment core with extraordinarily high sedimentation rates. Their data imply that times of minimum meltwater supply correlate with periods of cooling. This does not sound so alarming, as cooling is expected to halt melting. But the point is that, according to the conveyor-belt hypothesis, the halt in meltwater flow should result in warming, by starting off salt buildup and sinking of surface waters with the subsequent inflow of warm surface waters from the south.

This theory had already been challenged by two palaeoceanographers, Jansen and Veum⁷ from Bergen, who used microfossil carbon isotope data (a reliable tool for reconstructing deep water circulation) to suggest that deep convection continued in the North Atlantic even during the Younger Dryas cold period. Apparently, this convection failed to draw warm waters from the south into the polar North Atlantic. Now Veum *et al.*² use another record from a coring site further north to show that ventilation of the deeper water column in the Norwegian-Greenland Sea was probably as vigorous during the Younger Dryas as it is today. Their first carbon isotope record looked somewhat spurious, but with the new data one has to take this conflicting evidence seriously.

It is unlikely that Lehman and Keigwin are off the track because, first, the very high sedimentation rates of 5 metres per 1,000 years at their core site (compared with 2–5 centimetres per 1,000 years at open-ocean core sites) rule out the possibility of significant

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stratigraphical distortions; and, second, their records are well-dated and are correlated directly with similar climatic evidence drawn from the Greenland Dye-3 ice core. The dramatic short-term climatic oscillations documented in the ice core⁸ affected the regional climates all the way over to Northwest Europe.

The combined deep-ocean oxygen and carbon isotope data shown by Veum *et al.* imply that large parts of today's subpolar North Atlantic froze over during the Younger Dryas. The forming sea ice rejects salt, so increasing surface-water salinity, which apparently resulted in a density flux large enough to ventilate the deeper water column at least as effectively as today. Clearly, this evidence must be confirmed. If the data can be reproduced at other coring locations and still show that open-ocean convection occurred even during the coldest periods of the Younger Dryas — when

the conveyor belt was supposedly switched off — climate modellers will have to deal not only with the on and offs of the conveyor but possibly also with switches between cold and warm conveyors. The correlation of core data from the Norwegian–Greenland Sea with those from the shallow North Atlantic seems to imply that during the Younger Dryas the cold conveyor belt operated at shallower depth than today's warm belt. The driving force behind the cold belt would be a gradual buildup of salinity during sea-surface freezing, which would resemble the mechanism which starts off deep convection in today's Southern Ocean⁹. This factor is not accounted for by the concept of salt transport through heat and vapour flux^{4,5} and, frankly, it sounds rather far-fetched. Yet the data exist and must be confronted.

An obvious shortcoming of the conveyor-belt/salt-flux hypothesis is that

it deals with mean-ocean conditions and North-Atlantic mean flow rates. Thermohaline circulation is driven by regional and seasonal anomalies in the ocean's temperature–salinity field. The main sites of convection which feed today's conveyor belt are located in very restricted areas in the Norwegian–Greenland Seas and the Labrador Sea. In these areas convection occurs mainly during winter time. One may argue that this dependency on sinking of water masses in restricted provinces would make the conveyor belt even more vulnerable to large-scale meltwater input. Still, it seems that even during times of highest meltwater input immediately before and after the Younger Dryas, the deep North Atlantic was better ventilated than during the Younger Dryas¹⁰, which may have been meltwater-free⁶.

It seems critical to finding a solution for this circulation puzzle to determine

Chronology of Younger Dryas research

LATE 1970s TO EARLY '80s Dominance of polar foraminiferal populations implies nearly full-glacial conditions during the Younger Dryas which may be caused by rapid meltwater injection during the initial disintegration of continental ice sheets^{15,16}.

1987 NOVEMBER Geochemical evidence arises for extremely low ventilation of the deep North Atlantic during the Younger Dryas, implying a shut-down of water-mass sinking in the North Atlantic and a halt of the conveyor belt¹⁰.

1988 FEBRUARY Meltwater signals in sediment cores from the Gulf of Mexico and the subarctic North Atlantic imply a diversion of meltwater flow from the Gulf to the North Atlantic before and during the Younger Dryas. Poleward flow of warm waters in the conveyor belt was cut off causing the Younger Dryas climatic collapse around the North Atlantic³.

OCTOBER Stable isotope and planktonic faunal records from a Northwest Pacific sediment core show a cooling event synchronous with the Younger Dryas. The data suggest that the Younger Dryas was not restricted to the North Atlantic and that the cooling took in the high-latitude North Pacific¹⁷.

1989 MAY A drop in North Atlantic surface water temperature by almost 10 °C during the Younger Dryas is indicated by changes in the planktonic community structure¹⁸ and seems to be consistent with a conveyor-belt switch-off mode.

JUNE Geochemical tracers and airborne dust embedded in Greenland ice layers show the North Atlantic climate shifted 'back to normal' in less than 20 years at the end of the Younger Dryas⁸.

DECEMBER Growth rate curves of reef corals off Barbados point to strongly reduced meltwater inputs to the North Atlantic during the Younger Dryas. The hypothesis that increased meltwater discharge into the North Atlantic is the cause of the Younger Dryas comes under fire^{6,19}.

1990 FEBRUARY Benthic and planktonic isotope data from a northeast Atlantic sediment core imply increased deep water formation and ventilation of the deeper water column during the Younger Dryas, further undermining the meltwater hypothesis⁷.

AUGUST In a revision of the meltwater hypothesis, a salt

oscillator is designed which turns the conveyor belt on and off. The strength of the conveyor belt no longer depends on the rate of deglacial meltwater input, as the primary force is now heat flux and vapour transport⁴.

DECEMBER Numerical modelling of the salt oscillator suggests that North Atlantic surface salinity may fluctuate at periods of a few thousand years. The model does not test the sensitivity of salt oscillations to regionally variable meltwater inputs⁵.

Long time series of benthic isotope records show Younger-Dryas type climatic reversals during six deglacial transitions of the past 700,000 years. Each was supposedly accompanied by a temporary shut-down of deep-water production in the North Atlantic pointing to a switch-off of the conveyor belt¹³. Pollen records from southeastern Alaska document a cooling event which may have been coeval with the Younger Dryas; the Younger Dryas was a possibly hemisphere-wide event²⁰.

1991 JANUARY Deglacial climate breakdowns are observed in isotope records from two Sulu Sea sediment cores and are used to infer that the Younger Dryas was a global event caused by lowered atmospheric CO₂ concentrations²¹.

SEPTEMBER High-resolution palaeochemical records from the North Atlantic reveal periodic decreases in deep-water production during deglaciation at roughly the same periods as predicted from the salt-oscillation model. Decreased ventilation of the deeper water column during the Younger Dryas suggests the conveyor belt was switched off as North Atlantic surface salinities decreased, owing to meltwater input or cooling²².

1992 JANUARY Comparison of geochemical records obtained from North Atlantic and Southern Ocean sediment cores implies a significant influx of North Atlantic water masses to the Southern Ocean even during the Younger Dryas¹¹ — the conveyor belt remained switched on.

APRIL High-resolution geochemical and plankton data imply a rapid shut-down of circulation during the Younger Dryas in the North Atlantic¹, but benthic isotope data from the same region suggest that deep ventilation continued and was probably as vigorous as today².

whether thermohaline circulation truly behaves like a conveyor belt that shuts on and off, or whether the sites of convection shift between different locations as the salt oscillator pushes the subpolar North Atlantic from one mode to another. Perhaps the conveyor was never really switched off but operated at shallower depth. That way the deep Atlantic would not see the lower limb of the belt, but the North Atlantic would still be flushed with young waters. Then water from the North Atlantic could have reached the Southern Ocean even during the Younger Dryas, as indicated by the coherent development of geochemical proxies along sediment cores from both ocean basins¹¹. With more high-quality data from throughout the subpolar North Atlantic the evolution of

regional anomalies and their thermohaline momentum should become apparent¹². Special emphasis should be put on the other glacial-interglacial transitions of the past 700,000 years, which had their own Younger-Dryas type breakdowns¹³.

In view of impending global warming, progress in understanding this type of climate collapse is needed urgently. As recent work has shown^{1,8}, once the trigger has been pulled, North Atlantic climates can change dramatically within less than 50 years. And it seems¹⁴ that since the early 1980s a slow-down of deep convection in the Greenland Sea has already been under way. □

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T-CELL RECEPTORS

At grips with interactions

Alan F. Williams[†] and Albertus D. Beyers

DURING the 1980s the riddle of the T-cell antigen receptor (TCR) was solved, revealing a two-chain structure like an antibody Fab fragment with a binding surface likely to be constructed from hypervariable loops of the TCR V α and V β domains. This surface is proposed to interface with the top of the MHC molecule including foreign peptide bound in a groove formed by two α helices. The TCR, however, remained a receptor whose binding was inferred but not quantified.

This deficiency has now been rectified in two studies, one published in *Science*¹, the other reported on page 793 of this issue², where the binding affinity of TCRs has been measured. Matsui *et al.*¹ measured TCR binding affinity by determining the amount of soluble MHC class II antigen loaded with a relevant peptide (henceforth called 'MHCpep') that was needed to block binding of an anti-TCR Fab of known affinity to cell-borne TCR. From this data K_{equil} for the TCR was calculated to be about $2 \times 10^4 \text{ M}^{-1}$. Weber *et al.*² blocked T-cell responses with soluble forms of TCR and calculated a value of about 10^5 M^{-1} . Thus the independent studies are in quite good agreement, with both giving a low affinity of the order of the lowest known antibody reactions for binding. High affinities for antibody binding are due to somatic mutations as an immune response matures, and as the TCR does not undergo somatic mutation it seems that the current values should be taken as typical for TCR reactions.

Two other numbers are relevant in thinking about T-cell reactions. One is the likely time for 50% dissociation of monovalent TCR:MHCpep reactions

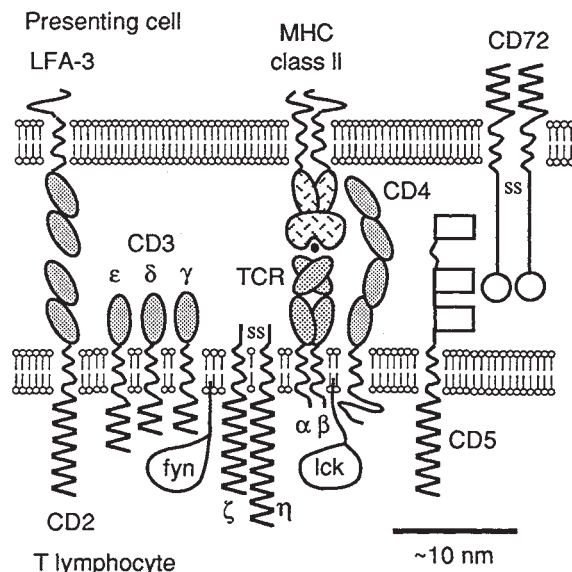
(the $t_{1/2}$ value). By comparison with a typical antibody this is calculated to be 0.3 seconds (the OX7 Anti-Thy-1 Fab has a K_{equil} of $3 \times 10^9 \text{ M}^{-1}$ and $t_{1/2}$ of 8,400 seconds; the TCR $t_{1/2}$ value is calculated from an affinity of 10^5 M^{-1} assuming an on-rate similar to OX7 Fab). The other relevant number is the number of MHCpep complexes that are needed to trigger a cell, which has been measured in the range of 50–300 molecules per cell^{3,4}.

What constraints do these numbers place on how the TCR may function? First, the binding energy between TCR and MHCpep must be a trivial factor in adhesion reactions between T cells and presenting cells. For example, a TCR contribution would surely be swamped by that of CD2:LFA3 binding alone, because there are about 5×10^4 molecules of CD2 per resting T cell and the K_{equil} for the CD2:LFA3 interaction has been measured at $2.5 \times 10^6 \text{ M}^{-1}$ (ref. 5). In fact, if the situation *in vivo* is considered, the concept of a T cell in one corner of a dish needing to find a presenting cell at another becomes nonsensical. *In vivo* a T lymphocyte in the blood leaves the bloodstream by interaction with the vascular endothelium and from then on will travel through a lymph node in a manner in which contact with other cells is always maintained. So the concept of bringing

cells together changes to one of triggering and guiding the movement of the T cell through the node.

Possibly, the molecules we refer to as adhesion molecules are the ones that determine the preferences of cell interactions and the pathways of movement. The T cell may move over cells that present no relevant antigen so that the recirculatory pathway is completed uneventfully. But if relevant MHCpep is presented, the TCRs bind and trigger the cell. Thus one can think of a cell moving along and receiving signals from its TCRs as it goes. If the TCRs achieve a sufficient firing rate, gross changes in the cell occur. Adhesion reactions are known to be strengthened after T-cell activation⁶, and one can imagine cell movement ceasing and the activated T cell engaging in further interactions with the presenting cell from which the triggering signals were received. The TCR can be expected to play a crucial role in all of this, not by virtue of the contribution that its binding energy makes to cell-cell adhesion but because it triggers the cell in a way that leads to qualitative changes in its state.

The quantitative parameters of the TCR reactions are also relevant to concepts of triggering by way of the TCR. It is generally thought that TCRs must be crosslinked to activate a T cell, yet how is this to occur through the TCR:MHCpep interaction? It seems most improbable that adjacent MHC molecules will display the same peptides, and to give a crosslinking signal two MHC molecules displaying the same peptide would have to be tethered together. With $t_{1/2}$ values of the order of seconds for the TCR reactions, and very small numbers of molecules involved, is



A T cell interacting with a presenting cell, showing molecules that are likely to exist in a T-cell activation complex for a T lymphocyte restricted by MHC class II antigen⁷.

it conceivable that a TCR:MHC_{pep} complex can wait around long enough for another such complex to find it and become crosslinked to it?

An alternative view is that crosslinking of TCRs does not occur, but that the key initial event is an interaction between the TCR complex and the CD4 or CD8 antigens that respectively bind to MHC class II or MHC class I antigens. The TCR complex is not alone at the cell surface but is in a loose complex with CD2, CD4 or 8, CD5 and the tyrosine kinases p56^{lck} and p59^{lyn} (refs 7,8).

The figure illustrates possible molecular interactions. The CD4 and CD8 antigens look much more like classical signal transduction molecules than do any of the TCR or CD3 chains. To a cell biologist a classical receptor has an extracellular binding domain, a transmembrane segment and a tyrosine kinase cytoplasmic domain, and this is exactly the case for CD4 and CD8 with the kinase being non-covalently attached⁹. It may be that when CD4 and TCR bind the same MHC_{pep}, the kinase domain is orientated to other cytoplasmic domains of the TCR multi-molecular complex such that a primary triggering event occurs. T-cell receptors and CD4 are present at cell surfaces at about 2×10^4 molecules per cell, and as they exist in preformed complexes it is imaginable that dual binding of these molecules to MHC_{pep} might frequently occur within the time in which a single TCR and MHC_{pep} are associated.

It might be argued that there are examples of T-cell triggering with cells that are CD4 or CD8 negative, and CD4 negative cells were used by Weber *et al.*². However it could be that the cells in question express CD4 at a few hundred molecules per cell and that this is sufficient for triggering on T hybridoma cells that are poised to respond. The key question is whether T-cell triggering involves crosslinking of the TCR molecules, or whether activation occurs by perturbation of a TCR multi-molecular complex by a single MHC_{pep}. □

'It is a matter of great regret to record that Alan F. Williams, Director of the MRC Cellular Immunology Unit, Sir William Dunn School of Pathology, University of Oxford, died of cancer earlier this month, shortly after completing this article. He was 46. Albertus D. Beyers is in the same unit.

Peter Mitchell (1920 – 1992)

PETER Mitchell, biochemist and Nobel laureate for chemistry in 1978, died from cancer on 10 April.

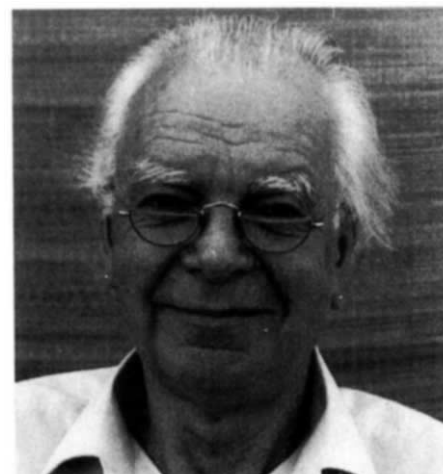
Mitchell's single-minded purpose was to understand the mechanistic relationships between the metabolism of cells and the transport of solutes across cellular membranes. Clearly they were related, but how? Metabolism could drive transport, and transport was essential for metabolism, but the links were not known. Metabolism, 1950s style, was bag-of-enzymes stuff, isotropic and re-constitutable in a test tube. Transport, or certainly its effect, was anisotropic. Mitchell reasoned that because ends could not be more anisotropic than their means, the membrane enzymes involved in both metabolism and transport would themselves need to be anisotropic, catalysing a reaction that is vectorial rather than scalar. Because such enzymes would be effecting both chemical transformations and solute translocation, he coined the word 'chemiosmotic' to describe the process that they facilitated.

The idea of chemiosmotic processes might have remained a curiosity in the literature but for Mitchell's dramatic exemplification of the concept. He proposed a richly predictive and highly successful mechanism for the manner in which the series of oxidoreductions within membranes, effected by mitochondrial respiratory chains or the photosynthetic apparatus of chloroplasts, could drive the otherwise thermodynamically unfavourable formation of ATP from ADP and phosphate. This idea cut through the impasse caused by abortive searches for the (non-existent) chemical intermediates that were then favoured.

The whole theory and its ramifications were set out in two privately published monographs, the unique 'grey books' of 1966 and 1968. They described the solution of a jigsaw, one where some of the parts had to be invented, and where the final picture was revealed only on its completion. Some of the pieces were already well known — the impermeability of membranes to most solutes, anions and cations; the need for specific proteins to provide specific diffusion pathways; the general distribution and, emergingly, the anisotropy of enzymes in membranes; the concept of membrane ATPases as cation pumps. The new pieces were the intrinsic proton-translocating activity of respiratory or photosynthetic oxidoreductions, the postulate of a reversible proton-translocating ATPase and, to complete the picture, reversible coupling of oxidoreductions and ATPase by a flow of protons. To all this was added postulated mechanisms for proton translocation driven by oxidoreductions and ATPases, experimental tests and analysis

of the thermodynamic requirements and predictions of the theory.

It was an amazing performance, set against a background of poor health, changes in career and marriage, and the need to act as his own architect, works manager and farmhand for at least two years (1963–1965) while he created his own laboratory and home at Glynn, a dilapidated country house in Cornwall. The support of his family was crucial for



Peter Mitchell — passion for science.

the success of these endeavours. Some notable exceptions apart, he received little support from grant-giving bodies or the major investigators in the field of oxidative phosphorylation. But he was essentially right. The chemiosmotic theory was tested, accepted and built upon.

Mitchell added further valuable ideas, notably for the functioning of ubiquinone, for coupling of ion movements to drive mechanical movements of flagellae, as the field at large moved to closer examination of the molecular mechanisms of chemiosmotic systems. So we arrive at the present, with crystallography, electron diffraction, recombinant DNA, site-directed mutagenesis — the tools available to the modern membranologist are of immense power, but the agenda is distinctly mitchellian.

He received numerous honours, yet remained modest, approachable and humorous. In *Who's Who*, his recreations are listed as "enjoyment of family life, home building and creation of wealth and amenity, restoration of buildings of architectural and historical interest, music, thinking, understanding, inventing, making, sailing". One begins to understand how his Cambridge PhD took seven years, what sustained his passion for science, what drove him on.

Peter Garland

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Proteases: to each his own

Dagmar Ringe

PROCESSING of propeptides involves the cleavage of one or more peptide bonds in the inactive precursor resulting in a smaller, sometimes fragmented, active protein. Very often these processing enzymes are unique to a particular propeptide and possess unusual specificity aimed at a limited number of cleavage sites. A paper on page 768 of this issue¹, and one earlier this month in *Science*², describe such a protease whose function is the processing of the propeptide from which the cytokine interleukin-1 β (IL-1 β) is derived. This specific proteolytic activation is part of a control mechanism designed to prevent random or unwanted side reactions, which might damage the cell by the activation of potentially dangerous molecules. Consequently, each activation process is highly specific for its own particular target.

Many proteins are synthesized as inactive precursors. The earliest identified examples belong to the eukaryotic serine protease family of proenzymes. Proteolytic enzymes are usually biosynthesized as somewhat larger inactive precursors known as zymogens, so preventing digestion of the tissues that synthesize them. Zymogens have very low enzymatic activity because the active site is distorted or blocked, preventing the productive binding of substrate. For digestive enzymes, specific tryptic cleavage at an arginine or lysine residue produces active enzyme in the case of trypsin and elastase, or produces an intermediate form which is processed further by autolysis to give active α -chymotrypsin. All of this processing takes place in the

duodenum in order to protect the pancreas from digestion, a sometimes fatal condition.

The prokaryotes seem to have developed a slightly different mechanism whereby autodigestion is prevented³. For instance, α -lytic protease is synthesized as a pre-proprotein which is activated autocatalytically. The precursor plays two important roles in the control mechanism, which protects the cell proteins from random attack by this protease. First, the precursor is inactive towards substrates, although it does have some low level of autocatalytic activity. But, more notably, the precursor is required for this protein to fold properly into its final three-dimensional form.

Early attempts to express mutants of this enzyme, designed to probe the functions of active-site residues, were unsuccessful unless the longer protein was expressed. Activation of the precursor is then required to produce the correct form of the final protease. These mutants have little catalytic activity, if any, so this requirement would seem to result in a conundrum. The problem has been solved by identification of the pro-region as a folding template which can be used separately to accelerate the formation of the active, native state⁴. Attempts to refold α -lytic protease *in vitro* resulted in an inactive, but folding-competent state with native-like secondary structure. Addition of the pro-region as a separate polypeptide chain resulted in recovery of active enzyme.

Biogenesis of the proteins and biologically active peptides required as chemi-

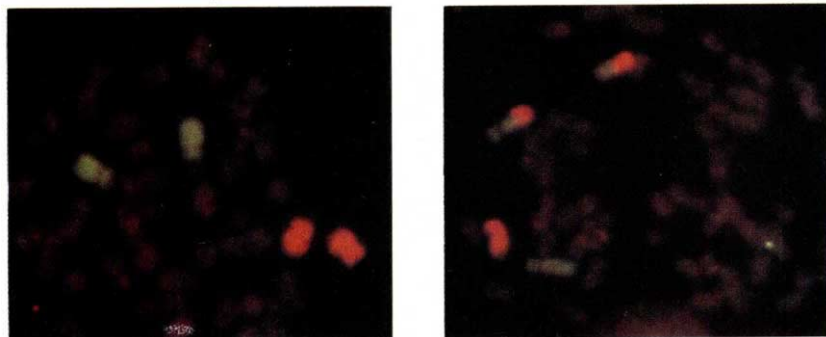
cal messengers for various forms of cell-process regulation is often regulated by a similar mechanism. Hormones such as insulin or β -endorphin are synthesized as prohormones or more complicated polypeptide precursors. The identification of the endoprotease Kex2 as essential for the processing of the precursors of yeast killer toxin and α -mating factor provided a clue to the possible nature of the enzymes responsible for processing hormone precursors⁵. From sequence analysis, Kex2 seems to be a subtilisin-like serine protease. Similar enzymes have now been identified in higher eukaryotic systems, which can reproduce all the main cleavages of a pituitary hormone precursor. This polypeptide, pro-opiomelanocortin, contains a total of seven different polypeptide hormones. Post-translational processing in the different lobes of the pituitary leads to the formation of various hormones with different physiological activities. Because the processing activities are different, different hormones seem to arise from different lobes, although all of them ultimately come from the same precursor. The processing proteases, PC2 and PC1/PC3, are distributed differently between the pituitary lobes, accounting for the distribution of the observed cleavage pattern⁶. The cleavage sites have a unique specificity, in that cleavage occurs between basic residues at Lys-Arg or Lys-Lys sites.

The control of hormone production, then, comes from a complex interplay of compartmentalization of highly specific processing proteases. So why is such a large polypeptide necessary? The answer may lie in a mechanism reminiscent of the α -lytic protease story. The specificity of the cleavage sites may be enhanced by secondary and tertiary structure of the polypeptide involved in presentation of cleavage sites to the proper protease.

The existence of a specific protease responsible for the activation of IL-1 β would seem to follow naturally from these considerations. Mature IL-1 β is a major mediator of inflammation. It is also regarded as a key hormone of the immune system, and has been implicated in septic shock and wound healing⁷. Attempts at control of this messenger and its associated activities have, up to now, centred on antagonists that resemble IL-1 β in sequence and structure⁸. Attempts to discover low-molecular-weight antagonists which might be capable of blocking the IL-1 β receptor without eliciting the normal response, and which would be suitable for oral therapy, have been unsuccessful. The particular importance of the new work reported by Thornberry *et al.*¹ and Cerretti *et al.*² is that it offers a new target for therapeutic intervention.

Interleukin-1 β is synthesized as an in-

Picture a specific translocation



MORE colour means more power in fluorescence *in situ* hybridization, a technique usually known by its acronym FISH or as chromosome painting. In its latest form, three-colour painting, the approach can be used to identify translocations between two specific chromosomes as caused, for example, by ionizing radiation; previously, 'banding' methods, which are slower, were used for the purpose. These two pictures come from the group of J.N. Lucas at Lawrence Livermore National Laboratory and illustrate the point. That on the left shows human chromosomes 4 (green) and 12 (red) in normal metaphase; that on the right translocation between the two chromosomes. The third colour involved is a propidium iodide counterstain.

T.L.

active precursor (pIL-1 β) almost twice as long as IL-1 β itself. The reason for such a construct fits perfectly with the considerations of hormone regulation discussed above. The full precursor is required for efficient folding and storage, releasing the mature, active protein on demand. That release mechanism comes in the form of a specific converting enzyme, IL-1 β converting enzyme (ICE), which is a highly specific protease that cleaves pIL-1 β at a single site, between Asp 116 and Ala 117. The enzyme is highly specific for aspartate in the P1 position, although its specificity must be more sophisticated because it does not cleave other proteins at analogous sites.

The enzyme itself is the product of a control mechanism of exquisite proportions. It has been classified as a cysteine protease by inactivation with known cysteine protease inactivators, although the primary sequence is not homologous to any known protein, including cellular cysteine proteases. I scanned the ICE primary structure against the three-dimensional structures of two members each from the serine, cysteine, zinc metallo- and acid protease families, using the method of Eisenberg *et al.*⁹, in the hope that the three-dimensional structure might contain information that the primary sequence does not. Eisenberg and colleagues' procedure attempts to thread the sequence of the unknown protein onto a known structure, and evaluates the quality of the resulting model. Sequence identity is not required. Using this method, I found no match for any known protease structure and the ICE sequence. Unfortunately, it has been my experience with this procedure that although a positive result is always significant, the absence of a match does not mean that no structural similarity exists.

The enzyme is biosynthesized as a pre-protein of M_r 45,000 which is processed into the active enzyme by cleavage at up to three sites. The specificity at these cleavage sites is highly constrained, requiring an aspartic acid residue at the P1 position. The precursor may be required for reasons similar to those applying in the case of α -lytic protease, namely mediation of folding and presentation of the correct cleavage sites to a processing enzyme⁴. The final enzyme is a heterodimer, both subunits of which are required for activity. Thus, dilution may be another element in keeping this protease under control.

An interesting analogy with Kex2 presents itself. There is a similarity in the organization of both ICE and Kex2 as determined from their primary structures. Both proteases have amino-terminal catalytic domains followed by carboxy-terminal domains that may be involved in localization as well as mod-

ification of activity or specificity. The carboxy-terminus of ICE has a cysteine-containing region (–CFYLFPGH–COOH) that could represent a site for farnesylation or geranyl geranylation if the last few residues were trimmed off. This cysteine is not the active-site cysteine, which is found in the other subunit. What is the possibility that this enzyme is anchored to the membrane, such as the Kex2 protease, thereby compartmentalizing its activity? The observation that the precursor is stable in blood is not necessarily proof against such a possibility.

A final element in the control of IL-1 β through control of ICE is the observation that intramolecular autoprocessing does not occur because the precursor is stable *in vitro* following translation. This implies that the precursor is inactive and the initial activation of converting enzyme requires proteolysis, mediated by yet another protease, before autocatalysis can continue the activation process —

SUPERFLUIDITY

Slowly unwinding in ^3He

P. V. E. McClintock

QUANTIZED vortices in superfluid ^3He wrapped around a smooth wire can slowly unzip themselves, precessing as they do so, according to a recent experiment by R. E. Packard and colleagues (R. J. Zieve *et al.* *Phys. Rev. Lett.* **68**, 1327–1330; 1992) at the University of California at Berkeley. This discovery was the unexpected bonus of an experiment which yielded an extremely accurate value of the quantum of circulation.

One of the most intriguing features of superfluids — such as liquid ^4He at 1 K or liquid ^3He at 10^{-3} K — is their characteristic property of quantized circulation, which implies that they cannot rotate in a conventional way like other liquids. When a vessel of superfluid is rotated, either the contents remain at rest (with respect to the fixed stars), or one or more quantized vortex lines appear in the liquid parallel to the axis of rotation. These consist of narrow cylindrical cores (much smaller for ^4He than for ^3He) around which the superfluid flows with a tangential velocity that decreases with distance from the centre. The theoretically predicted values of the circulation, which determine the strength of the flow, are nh/m_4 or $nh/2m_3$ for superfluid ^4He or superfluid ^3He , respectively, where h is Planck's constant, m_4 and m_3 are the atomic masses of ^4He and ^3He , and n is an integer (usually unity). The 2 in the denominator in the latter case reflects the fact that superfluidity in ^3He arises through a pairing of atoms.

Packard's group at Berkeley has set

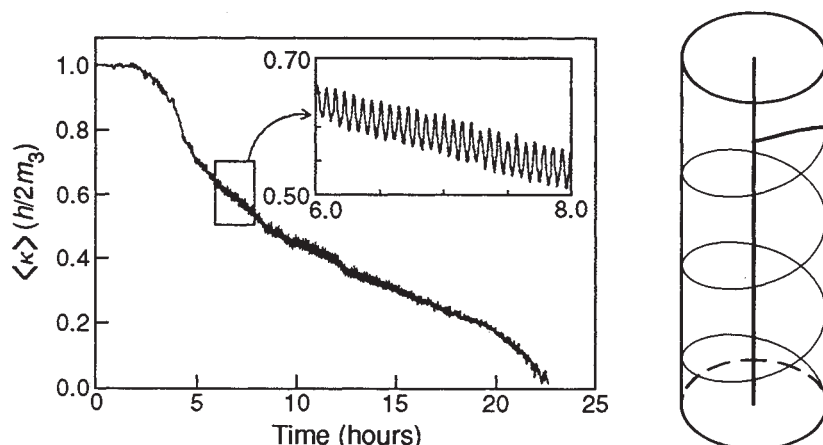
all of which sounds familiar to pharmaceutical chemists who have spent the past ten years trying to inhibit one or more of the consecutive processing enzymes in the angiotensin-activation pathway. So the identification of ICE and characterization of its properties presents not one therapeutic target for control of IL-1 β activity, but possibly two or more. To each his own. □

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about measuring the quantum of circulation κ in superfluid ^3He (expected to be $h/2m_3$) using essentially the same technique as W. F. Vinen employed for superfluid ^4He many years ago (*Proc. R. Soc. A* **260**, 218–236; 1961). Their experimental cell consists of a thin wire (16 μm diameter) stretched along the axis of a 2.96-mm-diameter brass cylinder filled with superfluid ^3He at a very low temperature (about 10^{-4} K) inside a rotatable cryostat. When the cryostat and cell are rotated fast enough, coaxially with the wire, it becomes energetically advantageous for a quantized vortex to form around the wire (with the wire replacing the vortex core). The rotation of the cryostat can then be stopped, leaving the vortex trapped in a metastable state around the wire. Its presence can be detected from the resultant rotation of the plane of vibration of the wire after it is 'twanged'; and the rate at which the plane of vibration rotates gives a measure of the rate of flow around the wire — that is, of the quantum of circulation, κ .

Some results obtained by Packard and colleagues for superfluid ^3He are plotted in the figure, and show that κ (divided by its predicted value) apparently decreases with time measured from the moment when the rotation of the cryostat was stopped: initially, κ is precisely equal to the predicted $h/2m_3$ but suddenly, after an hour or so, it starts falling steadily, finally reaching zero about a day later. Even more remarkably, super-



Left, the effective circulation around a wire in superfluid ^3He , measured as a function of time by Packard and colleagues. The data have been normalized by division by the predicted quantum of circulation ($h/2m_3$). Right, sketch to show how the vortex gradually unzips from the wire, which runs up the axis. The vortex surrounds the wire, from the bottom up to where it has unzipped, and then connects approximately radially to the surrounding brass cylinder. As the radial part of the vortex precesses under the action of hydrodynamic forces it dissipates energy, causing the vortex to shrink downwards along the wire and resulting in a helical motion of the point of attachment to the wall.

imposed on the overall decrease is a periodic oscillation (see inset).

How are these strange results to be explained? The occurrence of circulations apparently equal to non-integral multiples of the expected quantum of circulation is actually not new — something very similar was observed for superfluid ^4He by Vinen, who interpreted the effect as an indication that vortices can sometimes surround the wire, not completely as the theory assumes, but over only a fraction of its length. He assumed that the vortex left the wire at some point and that its end was attached (free ends would be unstable and are therefore not allowed) to the inner wall of the container. In Vinen's experiments, however, the measured circulations were stable (unless the wire or apparatus were banged) and did not change with time. Nonetheless, it seems likely that something rather similar is happening in superfluid ^3He . Packard and colleagues attribute the evident lack of stability to the larger core size of the ^3He vortices (1,000 times larger than those in ^4He), which means that their ends are much less likely than those of ^4He vortices to be stabilized by pinning to microscopic protuberances on the wire or the walls of the cell.

If one thus accepts the notion that an end of the vortex can easily slide over the surface of the wire or walls (and will therefore position itself so as to minimize the free energy), it becomes possible to interpret the results in the figure. Initially, when the rotation first stops, the vortex envelops the whole wire and the theoretically predicted value of κ is measured. After a while, however, some kind of random fluctuation occurs (and the geometry of the wire's attachment points will determine how easily it can

happen in practice) whereby one end of the vortex becomes separated from the wire and moves instead to the inner wall of the surrounding brass cylinder.

The situation is now radically different. Although the measured value of κ is initially still almost equal to the predicted one, because almost the whole of the wire is still enveloped by the vortex, the configuration has become inherently unstable: hydrodynamic forces cause a precession of the element of vortex connecting the wire to the wall, a process that is dissipative because the moving vortex core scatters the quasiparticles (thermal excitations) that are always present in the superfluid at finite temperature. The only place the dissipated energy can come from is the vortex itself, which correspondingly shrinks back gradually along the wire; as it unzips from the wire, its detached end will therefore precess in a helix (see figure). The circulation as measured in the experiment (on the assumption that the vortex surrounds the entire wire) will therefore seem to decrease continuously, finally reaching zero when no vortex remains, exactly as observed.

The authors argue convincingly that the fast periodic oscillation in the figure (inset) corresponds to the precession of the radial element of vortex round and round the cell. The effect arises because (serendipitously?) the wire was positioned slightly off axis in the cell: when the radial section of vortex becomes shorter, the section of vortex enveloping the wire has to lengthen slightly so as to conserve energy, causing the apparent circulation to increase momentarily. □

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Liquid vision

WHY are our eyes, those vital sense organs, so imperfect? About half of us need spectacles all the time; by middle age, nearly all of us need them for reading and other close work. Daedalus once advocated wearing an eye-patch from early youth, to save one eye from the fatigue and work-hardening of constant use. In middle age, you could take the patch off, and would then have one youthful eye for reading the small print. He now returns to the field with a subtler ophthalmic strategy.

He has been inspired by the strange medical fact that diabetics sometimes suffer from attacks of long-sightedness. Their sugar concentration is unstable. It is liable to fall, not only in their blood, but also in the vitreous humour of their eyes. Their eyes lose refractive power, making them long-sighted. Drunks also suffer notoriously from focusing problems, an effect which Daedalus similarly blames on the alcohol building up in their eye-fluids.

So DREADCO biochemists are devising a range of optical elixirs and vision-pills to adjust or fine tune the consumer's sight. Most chemical substances have a higher refractive index than the eye-fluid, so an elixir of short sight seems fairly feasible. Potassium chloride is already sold as a sodium-free substitute for salt, and Daedalus suggested making this elixir from more exotic and refractive salt analogues such as potassium bromide, rubidium iodide and so on. The DREADCO chemists rapidly overruled this simplistic notion; they are scanning the wider field of organic substances. They plan to synthesize a specialized range of biologically inert but powerfully refractive fluoro-sugars.

DREADCO's 'Bottled Short Sight' will be welcomed by scholars, watchmakers, and others who need to settle to long periods of close work. With luck, it could even last in the body for days. A carefully calibrated daily dose could then provide permanent ophthalmic correction. The happy consumer would at last be free of the curse of spectacles.

A short-lived formulation, rapidly degraded in the body, could provide useful bursts of short sight for entomologists, botanists and interpreters of insurance contracts. It might even be welcomed by lovers, whose vision is exercised at very close range and usually without their glasses. The ability to see one's beloved in sharp focus even in intimate proximity, should powerfully enhance the romantic experience. But less confident lovers may still prefer the traditional alcohol, to defocus their swain's vision and preserve the misty illusion of their fading charms.

David Jones

Glaciation and denudation rates

SIR — Summerfield and Kirkbride¹ question the general assumption that glaciation leads to an increase in denudation rates — an assumption that they attribute to Molnar and England^{2,3}, although this slightly overstates these authors' argument. We believe that Summerfield and Kirkbride's use of the work of Hicks *et al.*⁴ to support their thesis is inappropriate.

It is often stated that rates of erosion are higher for glacial than for nonglacial processes, typically on the basis of observations showing that sediment yields are higher in glacier-fed rivers than in comparable rivers not fed by glaciers^{5,6}. In departing from this conventional wisdom, Summerfield and Kirkbride refer to the work of Hicks *et al.*⁴, who found no significant difference in present-day sediment yield as a function of extent of glacier cover, based primarily on data from nine small schist basins in South Island, New Zealand. Summerfield and Kirkbride imply that the apparent lack of a present-day relationship between sediment yield and percentage glacierization contradicts the assumption that an increase in glacierization over time would result in an increase in denudation rates.

Such use of present-day spatial variations as surrogates for changes that might occur over time (an ergodic assumption) is a common approach in geomorphology, yet in this case it has serious limitations. Hicks *et al.*⁴ present data for basins with 0–77% glacier cover, and it is tempting to assume implicitly that this represents a population ranging from unglaciated basins to basins in something close to full-glacial conditions (and thus to extend their conclusions to changes during glacial/interglacial cycles). Clearly this is not the case because the valleys described⁴ are experiencing present-day interglacial (and in some cases paraglacial) conditions. During full-glacial times, these basins would have higher percentage glacier covers, but also far greater ice thickness, discharges, velocities, basal shear stresses and basal water pressures. Regardless of which predictive model for glacial erosion one chooses^{7,8}, it is clear that rates of glacial erosion are generally far higher during full-glacial conditions than a simple linear scaling with the change in percentage glacier cover would suggest.

Attempts to estimate current denudation rates are complicated by significant measurement errors and by an imprint of recent glacial history⁶ that is at present largely unquantifiable. In today's interglacial conditions it is possible that glacial erosion rates may be similar to nonglacial rates in some areas (as sug-

gested in ref. 4), but present-day relationships between denudation rate and percentage glacier cover are not appropriate surrogates for changes in denudation rate over time during glacial/interglacial cycles. At minimum it is clear that we cannot use the data in ref. 4 to refute the general idea of a positive association between periods of glaciation and enhanced denudation rates.

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Oscillating global temperature

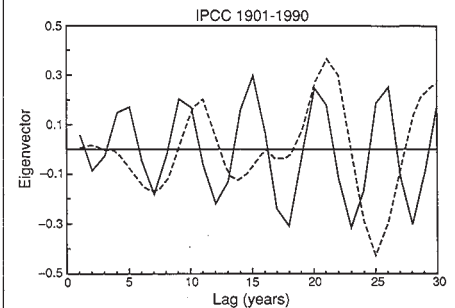
SIR — Ghil and Vautard¹ applied singular spectrum analysis to a global temperature data set and claimed the existence of a 20-year oscillation corresponding to a pair of high variance empirical orthogonal functions (EOFs). Subsequently, we² performed a sensitivity analysis considering various segments of that time series as well as of five other temperature records, and we concluded that the existence of the 20-year oscillation is questionable. We found that delineating a 20-year oscillation depends on including the (unreliable) early years of the records but not on the length of the records. Allen *et al.*³, using only the IPCC record, now say that when the early years are excluded, EOF4 and EOF6 exchange positions and that EOF3 and EOF6 form a 20–30-year oscillatory pair. Based on this observation, they conclude that we failed to prove our case against the 20-year oscillation.

Unfortunately, Allen *et al.* did not read our paper carefully. First, they say that we reported that the signature of the 20-year oscillation appears only if all 130 years are included. This is not correct. Our Fig. 3 clearly shows that, as long as the early years are included, the 20-year oscillation is present even when only 90 years are considered.

Second, Allen *et al.* create the impression that reproducing both Ghil and

Vautard's¹ and our² results constitutes a paradox. Ghil and Vautard considered the entire UK record, but we considered the length of that and five other records. Obtaining different answers does not therefore constitute a paradox.

Third, and most seriously, Allen *et al.* consider the last 110 values of the IPCC



EOF3 (solid line) and EOF6 (broken line) estimated from the lag-covariance matrix constructed from $M=30$ lagged copies of the last 90 (1901–1990) values of the IPCC consensus record of global temperature. The two EOFs do not represent oscillations of similar periodicity, and do not correspond to an oscillatory pair as Allen *et al.*³ suggest.

record and claim that EOFs 4 and 6 exchange places so that EOF3 and EOF6 constitute a 20–30-year oscillation. This claim is misleading. Careful examination of their figure (b)³ reveals that EOF6 has a period of about 15 years, whereas EOF3 has a period of about 25 years. Furthermore, these two oscillations start completely out of phase with each other and end up in phase. Allen *et al.*'s own figure shows that EOFs 3 and 6 are not in quadrature and do not form an oscillatory pair. Thus they mislead readers by suggesting that EOFs 3 and 6 do form an oscillatory pair. They are also wrong when they claim that EOFs 4 and 6 again exchange places when the last 90 years of the IPCC record are considered, even though their figure (a) indicates that the corresponding eigenvalues are not very similar. Our figure clearly shows that in this case EOF3 and EOF6 are not equal to each other, and there is no hint of a 20-year oscillation. The limited data and the fact that the eigenvalues may not be statistically significant make Allen *et al.*'s conclusions questionable.

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Species and speciation

SIR — In his valuable review of genetic differences that make us attribute individuals to different species, Coyne¹ repeated claims about sympatric speciation and the biological species concept (BSC) which, I believe, are incorrect, although widely accepted.

'Neodarwinism' is not limited to the "view that species usually arise as the byproducts of evolution in geographically isolated populations" (allopatric speciation). Sympatric speciation within one population is equally 'neodarwinian': both are caused by natural selection of genetic variation, although conditions of sympatric speciation are stringent (for example, only a few loci can be involved²), while isolated populations always diverge (but sometimes very slowly: *Platanus orientalis* and *P. occidentalis*, separated 25 million years ago, still produce fertile hybrids³).

The relative importance of allopatric and sympatric speciation has yet to be determined. However, the proponents of the latter have already "devised testable predictions to distinguish" it from the former. After allopatric speciation, new species differ in many loci because their ancestors were long isolated. During sympatric speciation, gene exchange continues, and new species retain similar allele frequencies at all loci, except the few responsible for reproductive isolation and ecological divergence². Striking genetic similarity between some sympatric species (for example, cichlids in Lake Victoria⁴ and trouts in Lake Sevan⁵) strongly suggests their recent sympatric origin (besides, in these cases there was no room for allopatric speciation).

Dogmatic adherence to the BSC causes much confusion. Of course, it is wrong to carve two species from a panmictic population or to pool into one species sexual forms which, if crossed, never have normal progeny. However, very distinct forms may produce fertile hybrids (including lions and tigers in captivity, and many species of Cyprinidae, Rosaceae and other taxa in nature). Perfectly fertile hybrids can flourish between populations, so different ecologically and morphologically that nobody would consider them the same species⁶, such as *Anemone fasciculata* and *A. speciosa* in the northern Caucasus⁷. Thus, speciation may precede the genetic isolation mechanisms and for a while depend solely on spatial separation caused by historical factors or different ecology (page 199 of ref. 3).

True, there are 'good' species, including ours, essentially homogeneous and drastically different from relatives, but life is not always that simple. To argue

about whether similar but separated populations are already different species is as futile as to discuss how many grains constitute a heap. To draw a sharp border in a gradual cline between two species is also impossible. Even sympatric forms are frequently isolated incompletely (for example species of *Papilio*⁸ and 'host races' of *Lochmaea*⁹).

In such cases we cannot discover species like new islands but, instead, face continuous diversity and have to describe it. Still, species, as a more or less distinct and genetically and evolutionary independent entity, is a useful concept, even when applied to asexuals, where ecological factors tend to create discrete forms (page 135 of ref. 10). For sexual organisms the degree of genetic exchange must be important for taxonomic decisions, although the Earth is not populated exclusively by 'good biological species'. This only makes genetics of speciation more exciting.

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SIR — Coyne¹ suggests that the new molecular mapping techniques should provide the data needed to construct models showing how genetic changes lead to speciation. We believe that such studies and models may be more fruitful if it is recognized that inherited epigenetic, as well as genetic, changes may underlie reproductive isolation, particularly in the early stages of speciation.

The importance of inherited epigenetic information is clear from studies of genomic imprinting^{2,3}. In many species, genetically identical genes or chromosomes function differently, depending on whether they were inherited from the male or female parent. Chromosomes from mother and father carry different, but complementary, imprints — different epigenetic information. One consequence is that mice that inherit both copies of a chromosome or chromosome region from the same parent are often abnormal. Similarly, mouse embryos constructed from two male or two female pronuclei do not complete development³. Although the genetic information they carry is complete and

adequate, the epigenetic information is not. To be viable, embryos must have the different, but complementary, epigenetic information from each parent. Incompatibility between the epigenetic information inherited from the parent species could be the cause of the inviability of some species hybrids⁴.

Exactly how chromosomes carry epigenetic information in chromatin structure is not known. In some higher organisms, patterns of DNA methylation are involved, but DNA–protein interactions are also important^{2,5}. Holliday⁶ has pointed out that errors in the transmission of such information (epimutations) are inevitable. Induced epigenetic changes are also possible⁴. The methylation patterns and functioning of particular genes in the mouse are known to differ from strain to strain³, and the accumulation of such differences could lead ultimately to inviability or sterility in hybrids between previously isolated populations. Studies of methylation patterns and gene activity in interspecific crosses would help to determine whether this is so. Unfortunately, *Drosophila*, the genus for which there is most genetic information about speciation, shows little or no DNA methylation.

Recognizing that information carried in chromatin may be important helps to explain some of the observations discussed by Coyne¹. Faulty chromatin structure has been implicated in some meiotic drive systems, and speciation resulting from cytoplasmic symbionts is associated with defective chromatin condensation. The disproportionately large effect of the sex chromosomes on post-zygotic isolation may be a consequence of the greater changes in chromatin structure that these chromosomes undergo during development, and Haldane's rule may be the result of the more marked chromatin restructuring that takes place in the heterogametic sex⁷. Because changes in chromatin structure seem to be involved in so many aspects of reproductive isolation, studying the heritable epigenetic information in chromosomes may be necessary if speciation is to be understood.

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Maxwell's other demons

Peter M. Harman

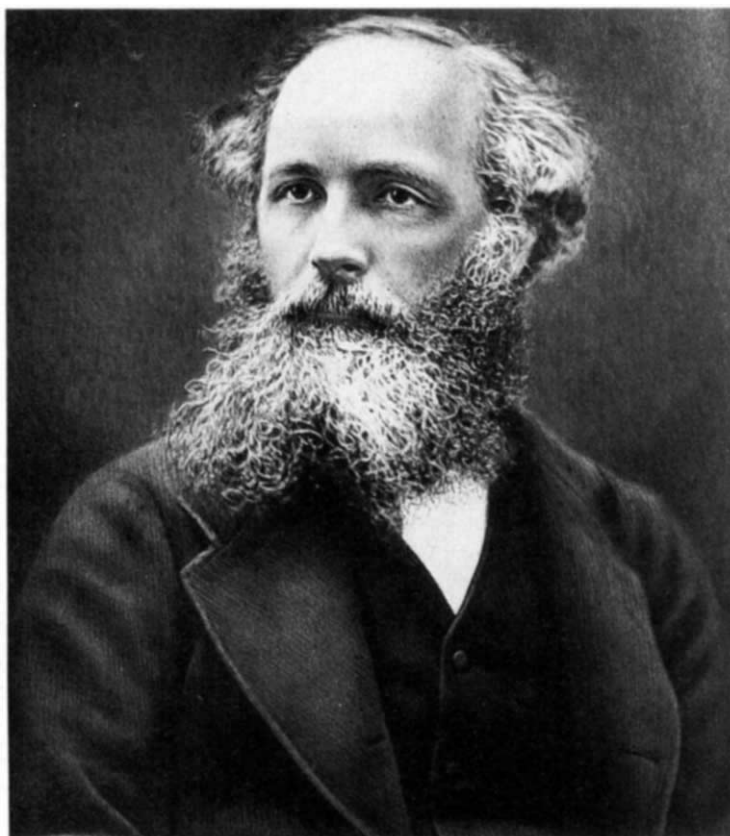
Innovation in Maxwell's Electromagnetic Theory: Molecular Vortices, Displacement Current, and Light. By Daniel M. Siegel. Cambridge University Press: 1992. Pp. 225. £30, \$49.95.

JAMES Clerk Maxwell's theory of the electromagnetic field ranks as one of the most important scientific achievements; on his field equations rest substantial elements of twentieth-century science and technology, from the theory of relativity to colour television. The study of 'Maxwell's equations' is essential for understanding physics and electrical engineering, and two of Maxwell's innovations have retained a central place in both teaching and historical exegesis: the displacement current, which completes the formal structure of the field equations, and the demonstration of the electromagnetic theory of light. Indeed, questions about the meaning and intelligibility of Maxwell's arguments behind these two innovations have been at the core of discussion about the origins and development of his field theory ever since Ludwig Boltzmann published commentaries on Maxwell's early papers on field physics in the 1890s.

The two innovatory ideas were first introduced in Maxwell's second paper on field theory, "On physical lines of force", published in four parts in the *Philosophical Magazine* in 1861 and 1862. It is this paper that forms the subject of Daniel Siegel's impressive book. The paper has proved to be very controversial, for over and above the general difficulties of unravelling its technical complexities and thematic subtleties, it contains specific and intriguing problems. Here Maxwell introduced his mechanical model of the ether, still the most famous image of nineteenth-century physics. Maxwell's hypothetical ether, constructed of a honeycomb of rotating molecular vortices with 'idle wheel' particles between the vortices, has long seemed an enigma.

It has been open to debate whether the model was intended merely to be an illustration of the physical ideas, or whether it shaped the physical theory

and, if so, exactly how. A variety of positions have been held by competent authorities: Pierre Duhem dismissed the model as merely illustrative, an adaptation of a model developed for electromagnetism to the case of optics, and as inappropriate to boot; whereas Boltzmann inclined to the view that the



James Clerk Maxwell (1831–1879): his mechanical ether model shaped his physical theories rather than merely illustrating them.

model shaped Maxwell's theoretical argument.

Siegel believes strongly that the ether model was determinative of Maxwell's argument in the paper "On physical lines of force". As he rightly emphasizes, Maxwell remained committed to the hypothesis of molecular vortices throughout his later career, while he noted the artificiality and provisional nature of the idle wheel hypothesis (the flux of these particles having been envisaged as a representation of the flow of an electric current). To counter critics of the intelligibility of Maxwell's ether model, Siegel establishes that the model depends on credible mechanical assumptions. The extension of the original

model to electrostatics was fundamental to Maxwell's theory: here he introduced the displacement current, and Siegel demonstrates that the often-vaunted supposed inconsistency of algebraic signs in Maxwell's exposition is resolved when the argument is seen as fully grounded on the mechanical model. This part of Siegel's work will be especially interesting to university teachers of physics.

On the formulation of the electromagnetic theory of light, Siegel shows that the argument rests on mechanical approximations in adjusting the parameters of the mechanical ether model. In that way, the propagation of elastic waves in the ether could be interpreted in electromagnetic terms. The discovery that the velocity of propagation of these waves was equal to the velocity of light was, as Maxwell reported in letters to Michael Faraday and William Thomson (Lord Kelvin) in late 1861, truly a discovery, and was independent of the manipulation of the parameters of the mechanical ether model. This result is, as Siegel points out, a prime example of a major theoretical innovation based on little experimental evidence. Siegel has successfully rescued Maxwell from charges of theoretical fudging. His book is therefore an important contribution to the history of physics.

Siegel puts Maxwell's approach to theory construction in the context of Cambridge methodology (where Herschel and Whewell emphasized comprehensive fundamental theory), Scottish scepticism towards untrammelled hypotheses (hence Maxwell's caution in presenting the ether model as "provisional" and "awkward"), and the field-primacy theory of Faraday. This interpretation is convincing, but Siegel makes no serious attempt to place the argument of "On physical lines of force" within the broader context of Maxwell's physics. Although the gain in precision and depth of analysis must be applauded, the picture that emerges does not of course characterize the full thrust of Maxwell's work on field theory. The focus here is on the innovative nexus, on the displacement current and the electromagnetic theory of light. To concentrate on these questions is fully justified; yet Siegel's thoroughly convincing study stands, I believe, at the beginning of a truly

historical analysis of the development of Maxwell's field theory.

Siegel's achievement may be seen as having brought the Maxwellian disputes to a close. The task now facing historians is to comprehend the diversity and relationship of the different modes of representation that Maxwell advances in his papers on field theory; and to understand the synthesis attempted in his

Treatise on Electricity and Magnetism (1873) in terms of the innovatory mathematical physics. To place this intellectual titan's electromagnetic theory within the wider pantheon of his physical worldview is a challenge for future scholars. □

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In sickness and in health

Arnold S. Relman

Health in the Headlines: The Stories Behind the Stories. By Stephen Klaidman. Oxford University Press: 1991. £19.50, \$24.95.

THE public's appetite for news about science and medicine seems boundless and the popular media devote an increasing amount of attention to the two subjects. Sometimes, the news is straightforward and, except for the technical details, reporters have no special problem in telling it. But often the issues are complex and controversial and, to make things even more difficult, of great importance to people's health. Although the public clearly has a large stake in knowing the facts, they are usually not at all obvious, or they are strongly debated by the experts.

Stephen Klaidman, an experienced journalist, recounts "the stories behind the stories" of seven health-related and controversial issues that have dominated the news headlines at various times over the past decade or two. He tells us in

great detail how the media handled stories about the toxic pesticide ethylene dibromide (EDB); contamination of basements with radon gas; AIDS; the diet/cholesterol/heart disease controversy; the health hazards of nuclear power; the greenhouse effect and the consequences of global warming; and the effects of smoking on health.

In separate chapters, Klaidman painstakingly chronicles the events and personalities involved in bringing each issue to the public's attention. Each story is unique; there are only a few common threads. Reporters, we are told, are not always as well informed as they should be; editors and publishers are sometimes biased by commercial considerations or by their connections with powerful political figures. And, underlying it all, is the difficulty of making readers, who are often scientifically illiterate, understand the subtleties of epidemiological methodology and the limitations of results that are based largely on statistical associations.

The book is richly detailed. Klaidman is a careful reporter and he writes well. Disappointing, though, is his limited discussion of basic questions. How can reporters be trained to deal better with complicated science and health stories? Are there some general guidelines that would help both reporters and the public to understand the strengths and weaknesses of epidemiological research? (An editorial by Marcia Angell in the *New England Journal of Medicine* of 20 September 1990 provides an excellent start.) Should news stories about scientific research be handled differently from the usual news reports, and should there be different criteria for publication?

These and many other questions are raised in the book, but readers will look in vain for answers. Perhaps Klaidman can be forgiven because the answers are not obvious and will emerge only after much more discussion and reflection. In any case, he has written an interesting and informative book which breaks new ground in an area that clearly deserves a lot more attention. □

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Belief in GAD

Michael Morgan

The Lopsided Ape: Evolution of the Generative Mind. By Michael C. Corballis. Oxford University Press: 1992. Pp. 336. £18.95, \$24.95.

A DOMINANT theme in philosophy, theology and science has been the nature of the difference between humans and other animals. Is the difference one of substance or just degree? Humans are distinguished from their nearest living relatives by attributes such as the possession of spoken and written language, cerebral hemisphere asymmetry for speech, a strong hand preference, skeletal and sexual adaptations to an upright gait, a considerable capacity for innovation in technology, science and art, and the absence of large canine teeth. It is tempting to link these attributes together into a panoptic theory of human evolution, and psychologists in particular have been drawn to cerebral asymmetry as possibly the most important underlying feature. Many theories have been proposed in which either speech asymmetry or handedness and the use of tools are the basis of human uniqueness. These theories attempt to explain why all the attributes peculiar to humans are associated, although it is probably fair to say that psychologists have not had a great deal to say about the canine teeth.

Michael Corballis, an authority on human brain asymmetry, argues that humans were created by GAD about two million years ago. GAD is the generative assembling device, a hypothetical left-hemisphere neural structure responsible for language, praxis (the ability to carry out complex series of voluntary motor acts), and the ability to form visual representations of objects consisting of several different parts, such as a telephone or chair. GAD evolved in relation both to tool use and technology, and to vocal language, the connection between the two being so intimate that it is difficult to separate them. GAD is specific to the dominant hemisphere (usually the left) and the minor hemisphere remains essentially pre-GADian and like that of animals. Popular hemispherology is not altogether incorrect in supposing that the left hemisphere is analytical and logical while the right is more 'holistic' and intuitive.

Corballis begins with a wide-ranging and erudite review of relevant findings in physical anthropology, psycholinguistics, neuropsychology and visual perception. He then reaches the core of his argument, introducing the concepts of praxis and generativity. Probably nobody has

New Journals issue

This year, *Nature's* annual new journals review supplement will appear in the issue of 1 October. Publishers and learned societies are invited to submit journals for review, taking note of the following criteria:

■ Journals that first appeared during or after June 1990 and issued at least four separate numbers by the end of April 1992 will be considered.

■ Journals covering any aspect of science are eligible, although those dealing with clinical medicine, engineering and pure mathematics are excluded, as are publications of abstracts.

■ Frequency of publication must be at least three times a year. The main language used must be English. Translation journals in English are, of course, eligible.

■ Deadline for submission is the end of May.

When submitting journals for review, please send at least four different issues (the first, the most recent and any two others) of each title, together with full details of subscription rates (personal and institutional) and frequency of publication, to: Peter Tallack, *Nature*, 4 Little Essex Street, London WC2R 3LF, UK. For further information please telephone Peter Tallack on 071-836-6633 (011-44-71-836-6633 from the United States), extension 2414.

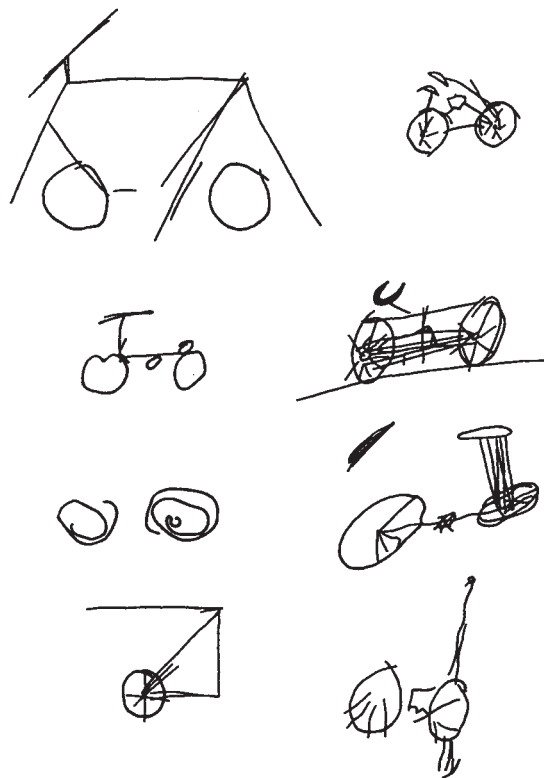
ever written this sentence, or the previous one, before, which makes the point that language generates infinite novelty by combining basic units into sequences. Corballis argues that skilled manual activity or praxis (from Greek, *praxis*, action) does the same. Patients with apraxia find it difficult to do simple tasks, such as picking up and hammering a nail. Both apraxia and aphasia (loss of generative speech) are associated with damage to the left hemisphere (although by no means necessarily together), and this constitutes the principle neuropsychological evidence for GAD. This part of the argument has been heard before, but Corballis extends it in a novel direction, arguing that human visual perception is unique in being able to generate and decompose objects consisting of many different parts. This too is a left-hemisphere skill under the control of GAD.

The perceptual theory relies heavily on I. Biederman's "Geon" theory, according to which objects are composed of geometrical primitives such as spheres and cylinders, combined in different ways. The theory, which may be seen as greatly influenced by computer graphics, is controversial and seems to apply much more readily to human artefacts than to trees and terrain. But perhaps this is the point: is today's technology, with its inexhaustible novelty, an extension of the generative skills of language?

Corballis's argument is stimulating, but is not without its problems. I wonder, in the first place, if there is any evidence that the left hemisphere is innately specialized for praxis, rather than acquiring its superior motor skills through biased practice. That we are born with a right-hand preference is beyond doubt, but is the right hand innately any more skilful than the left? The crucial evidence would come from thalidomide victims who have either a left or right hand: would right-handers be on average more skilful than left-handers? The only study of congenital unilateral aplasia that I know of found no difference. Further evidence against innate differences in skills comes from a recent study by K. J. Connolly and D. V. M. Bishop (*Neuropsychologia* 30, 13; 1992). They studied preferences and manual skills in children from Papua New Guinea, many of whom were unfamiliar with implements such as

pencils or spoons. Their preferences were as marked as those of English children, but the degree of skill asymmetry in the task of moving and tapping pegs was much smaller. This is exactly what we would expect if the greater skill of the right hand is due to practice rather than to an innate GAD in the left hemisphere.

I find even greater difficulty with the evidence that Corballis cites to support a left-hemisphere GAD for the visual representation of objects. Do other animals really have a problem decomposing ob-



Bicycles drawn from memory by patients described by McFie and Zangwill. Drawings on the left and right are by patients with left- and right-hemisphere damage respectively.

jects into parts? Studies of rats and pigeons seem to indicate that this is exactly what they are good at, and the defence that these animals decompose into 'features' rather than 'primitives' is weak. On the neuropsychological side, J. M. McFie and O. L. Zangwill's classic studies of parietal damage (*Brain* 83, 243; 1960) showed that objects drawn by patients with right-hemisphere parietal damage had parts that although well represented, were depicted inaccurately in their spatial relation to other parts; this is surely the reverse of what would be expected from the theory if GAD were in the left hemisphere. Corballis also notes that "mental rotation" is impaired by damage to the right hemisphere; saying that this represents loss of a "holistic" skill rather than of an ability to rearrange the parts seems to

Hawking revisited

The 'Hawking industry' continues apace with the release of a documentary film of *A Brief History of Time*, which itself now boasts sales of more than five and a half million copies in 30 languages. Directed by Errol Morris and accompanied by an original score by Philip Glass, the film, which takes the name of the book, uses interviews with Hawking's family, friends and colleagues to trace the development of his work and life. The result — better described as a brief history of Stephen Hawking — is released on video by Palace Pictures and will be shown on television in the United Kingdom on Channel 4 on 3 May; it should hit cinemas in the United States in July. And for those wishing to read the book of the film of the book comes *Stephen Hawking's A Brief History of Time: A Reader's Companion* (published tomorrow in the United Kingdom, and in June in the United States; Bantam, £16.99, \$23). The book follows the film closely, borrowing interviews and stills from the documentary, and contains a glossary and further scientific discussions by Hawking. Also issued in the United States in July is John Gribbin and Michael White's biography *Stephen Hawking: A Life in Science* (Dutton, \$23; for a review see *Nature* 356, 25; 1992). Finally, of related interest is last month's issue of *The Cambridge Review: A Journal of University Life and Thought*, in which, among others, John Polkinghorne and Malcolm Longair reconsider *A Brief History*, reflecting on the book's ideas and on the state of popular science writing today. Included is a response by Hawking. The journal is published by Cambridge University Press.

New in paperback

- *The Genocidal Mentality: Nazi Holocaust and Nuclear Threat* by Robert Jay Lifton and Eric Markusen. Published by Macmillan, price £12.99. For a review see *Nature* 350, 200 (1991).
- *The Politics of Evolution: Morphology, Medicine, and Reform in Radical London* by Adrian Desmond. Published by The University of Chicago Press, price \$22.95, £15.95. For a review see *Nature* 344, 392 (1990).
- *Burning Bush: A Fire History of Australia* by Stephen J. Pyne. Published by Owl Books, price \$15.95. For a review see *Nature* 350, 539 (1991).
- *The Age of Intelligent Machines* by Raymond Kurzweil. Published by MIT Press, price \$22.50, £15.95. For a review see *Nature* 348, 399 (1990).

Mathematica for Windows

Wolfram Research's *Mathematica 2.0*, the revolutionary general software system for doing mathematics by computer, is now available for use in the Microsoft Windows environment. *Mathematica* handles numerical, symbolic and graphical computations and has a built-in programming language (for reviews see *Nature* 336, 319 (1988) and 351, 437 (1991)). The Windows version costs \$955.

me to be a case of special pleading.

The Lopsided Ape is nevertheless an excellent read, enlivened by many anecdotes, historical details and jokes. On the problems of the speech-deprived right hemisphere, Corballis (a New Zealander) remarks in a footnote that the disadvantages are well understood by his fellow countrymen "who also share a hemisphere with a more talkative neighbour". □

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Birth of a new frontier

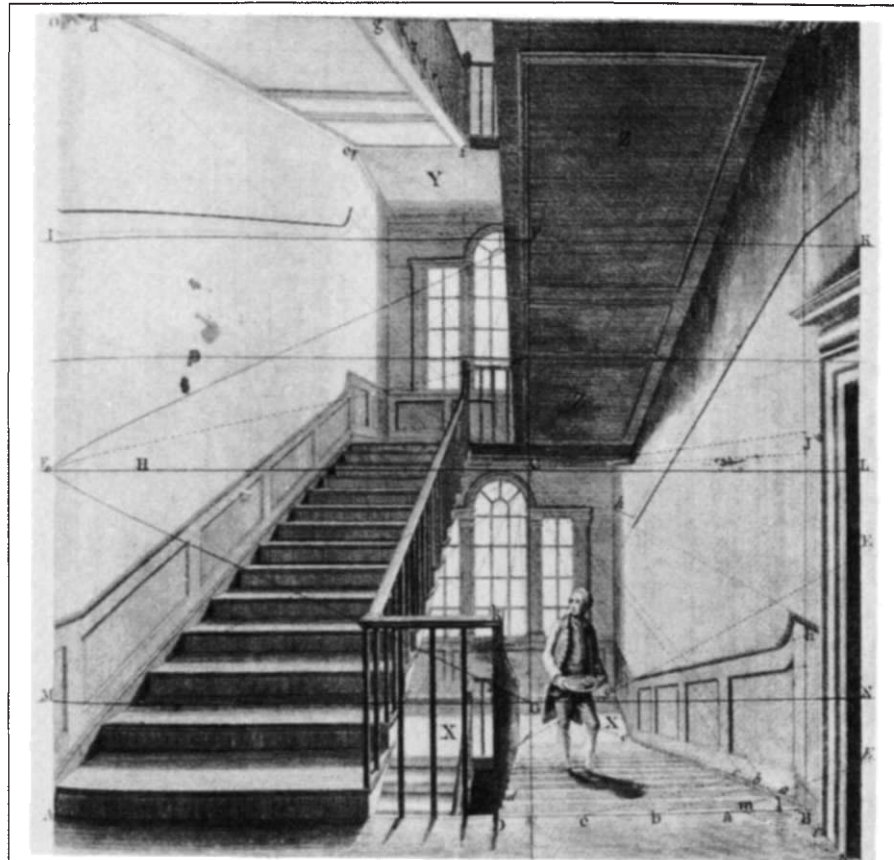
David J. Miller

The Meson Factories. Edited by Torleif E. O. Ericson, Vernon W. Hughes and Darragh E. Nagle. University of California Press: 1992. Pp. 870. \$75.

THE obvious frontiers of knowledge are not the only places where new scientific disciplines grow. In the period covered by this substantial volume of reprints and commentaries (the mid-1970s to the late 1980s), the high-energy frontier of particle physics had already reached hundreds of gigaelectronvolts, but the impressively diverse work described here is all at or below a few hundred mega-electronvolts, based particularly on the programmes of three meson factories, a proton linear accelerator at Los Alamos (LAMPF), an isochronous ring at Zurich (formerly SIN, now PSI) and an H⁻ cyclotron in Vancouver (TRIUMF). These factories are distinguished from the ubiquitous cyclotrons of the 1950s and 60s by their high beam currents (0.1–1 millamperes), which have made whole new classes of study possible.

The 50-page introduction gives the context in which the machines were built. Although the editors pick out their own list of outstanding results, many readers will find equally valid alternative lists from their own browsing through the reprints (121 in all), which cover the machines themselves (9), particle physics (27), nuclear physics (35), atomic physics (26), condensed matter (18) and applications (6).

In the section devoted to particle physics we are reminded that the standard model of electroweak theory has been tested as rigorously in muon (μ -meson) and pion (π -meson) decays as it has in neutrino scattering or Z^0 and $W^\pm$ particle physics. There are classic papers on the mean lifetime of the positive muon (the baseline for all fits to the electroweak parameters) and on the limits to



Perspectival study of a staircase, from Malton's *A Complete Treatise on Perspective* . . . (1779). The plate is taken from Martin Kemp's beautifully produced book *The Science of Art: Optical Themes in Western Art from Brunelleschi to Seurat*, now published in paperback by Yale University Press, price \$35, £19.95.

forbidden decays of muons, for example to an electron and a photon. High-precision pion–nucleon and nucleon–nucleon scattering experiments measure the low-energy scattering amplitudes and establish the degree of charge-symmetry breaking.

There are two papers on charge-symmetry breaking among the nuclear-physics reprints, and much detailed work on spin-dependent scattering amplitudes is described. Pion double-charge exchange is covered by six reprints.

It is in the atomic-physics section that totally new disciplines emerge most clearly. Muonium (an artificial electron–muon 'atom') provides a new laboratory for the precise study of quantum electrodynamics, without the uncertainties caused by the structure of the proton. There are reports of muonium's production into vacuum and the measurement of its hyperfine splittings. Its Lamb shift gives a new precise value for the fine structure constant and for testing model calculations. Muonic atoms (in which a Muon replaces an electron) are another new laboratory. The muon's small orbit in heavy atoms means that vacuum polarization effects can be tested more rigorously than in any other system.

Perhaps the most important application of low-energy meson beams will

turn out to be muon-catalysed fusion of deuterium and tritium. The volume includes the theoretical paper by Gerstein and Ponomarev showing that resonant formation of an excited muonic molecule should allow each negative muon to catalyse roughly 100 fusions; also reprinted are the first experimental results indicating this level of catalysis.

Many of the reprints on condensed matter concern applications of the muon spin-rotation technique, although there is also a paper on the use of the LAMPF pulsed thermal neutron facility to study a high-temperature superconductor. Applications are covered by five reprints on medical physics, and one on radiation damage in aluminium.

Students starting PhD studies in any of the fields covered — whether at a meson factory, at ISIS, at Grenoble or elsewhere — should be required to read the whole volume to see the context in which they will be working. But it is a good browse for any physicist, and something to put on the library purchase list. (It has the advantage of being too heavy to be easily stolen.) □

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Sudden changes in North Atlantic circulation during the last deglaciation

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Sudden changes in the flow of warm Atlantic surface waters into the Norwegian Sea occurred frequently during the last deglaciation, typically involving shifts in sea surface temperature of $\geq 5^\circ\text{C}$ in fewer than 40 years. These led to equally large and rapid changes in atmospheric temperatures, and to shifts in Atlantic deep thermohaline circulation and ice-sheet melting rates.

THE last glaciation (70–10 kyr BP) is marked in Greenland ice cores by strong oscillations in oxygen-isotope content ($\delta^{18}\text{O}$), which are widely believed to reflect sudden changes in the temperature of precipitation over the ice sheet¹. Annual layer counting through the most recent of these indicates that a warming of $\sim 7^\circ\text{C}$ occurred within a 50-yr period during the transition from the Younger Dryas cold phase (~ 11 –10 kyr BP) to the present interglacial². These oscillations occur both too frequently and too abruptly to have been directly forced by long-term changes in incident solar radiation, and are instead attributed to sudden changes in the rate of thermohaline overturn in the North Atlantic, which effectively draws heat from temperate latitudes towards the pole^{3,4}. According to numerical models, overturn in the North Atlantic may be damped by small increases in freshwater flux at the surface, and the circulation may even oscillate between 'on' and 'off' modes^{5–8}, thus strongly influencing climate in the circum-Atlantic region on short timescales⁹. But it has not been possible to verify the oceanic origin of these sudden climate shifts in the stratigraphic record of the deep sea because of the difficulty in resolving events that span less than a few hundred years.

Here we present new data on the timing, rates and cause of circulation change in the North Atlantic for the last deglaciation. This is based on the study of faunal and $\delta^{18}\text{O}$ variations in a sediment core from the Norwegian Trench, where rates of deposition are comparable to those in the Greenland ice cores. Our

results demonstrate that sudden changes in the surface circulation occurred frequently during the deglaciation, often corresponding to variations in summer sea surface temperature (SST) of $\geq 5^\circ\text{C}$ over a few decades. The history of these changes closely follows the $\delta^{18}\text{O}$ variations in Greenland ice cores for the past 14 kyr, confirming that shifts in air temperature over the ice sheet were caused by changes in the poleward flux of ocean heat. Sea and air temperature changes appear to be correlated with shifts in the production rate of Lower North Atlantic Deep Water (LNADW). Because of the exceptional rate of heat loss during its formation, we argue that LNADW is the pre-eminent deep water mass in controlling surface climate in the circum-Atlantic region. We show here that changes in LNADW, and associated shifts in air and sea temperatures, strongly influenced global and local ice-sheet melting histories.

High-resolution faunal records

Down-core and synoptic changes in assemblages of planktonic foraminifera have been used routinely to chart past movements of the boundary between polar/subpolar and temperate water masses (the polar front), and ocean response times in the North Atlantic¹⁰. Today, relatively warm Atlantic surface waters flow northwards and enter the Norwegian Sea basin between the Faeroe and Shetland Islands. As a result, subpolar foraminifera now dominate faunal assemblages in the southern and eastern part of the basin¹¹ (Fig. 1). During the last glaciation, polar fauna (containing almost exclusively the sinistrally coiled (*s*) form of *Neogloboquadrina pachyderma*) spread throughout the basin, indicating that the polar front had advanced into the North Atlantic and that the poleward flow of warm waters was cut off¹². The polar front began to retreat from its full glacial position at ~ 13 kyr BP, then advanced towards the Equator between ~ 11 and ~ 10 kyr BP during the Younger Dryas, before finally retreating to near its present position¹³. Radiocarbon dating of faunal changes constraining deglacial movements of the polar front suggests that it swept between these extremes in 400 yr or less¹⁴, a rate still 10 times slower than that implied by the Greenland ice core record. But it is not known to what extent

FIG. 1 Map of the North Atlantic region showing the positions of the modern and reconstructed last glacial maximum (LGM) polar fronts¹², and the location of deep sea and ice cores mentioned in the text.

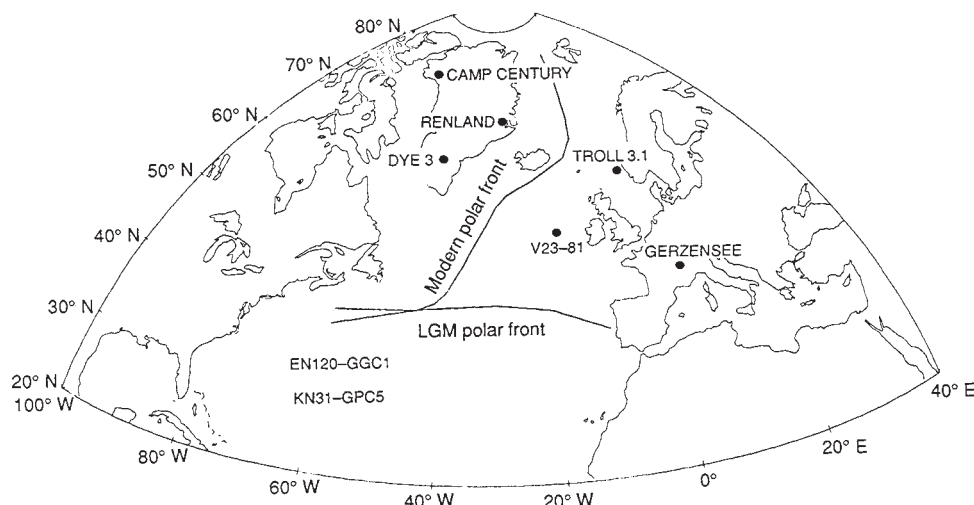


TABLE 1 Data from core Troll 3.1

Depth (cm)	Age* (kyr BP)	N.p.-s (%)	Benthic $\delta^{18}\text{O}$ (‰)	N.p.-s $\delta^{18}\text{O}$ (‰)	N.p.-d $\delta^{18}\text{O}$ (‰)	AMS ^{14}C age†	Depth (cm)	Age* (kyr BP)	N.p.-s (%)	Benthic $\delta^{18}\text{O}$ (‰)	N.p.-s $\delta^{18}\text{O}$ (‰)	N.p.-d $\delta^{18}\text{O}$ (‰)	AMS ^{14}C age†
19.75	1.49	0.51	2.23	1.57	1.26	1,490 ± 60	425.00	10.59	100.00		3.18		
30.00	1.87	0.24			1.3		445.00	10.69	100.00	3.80	2.96		
40.00	2.24	0.40			1.20		463.00	10.77	95.65	3.87	2.90		
50.00	2.61	1.15			1.10		464.00	10.78		3.87			
60.00	2.98	1.51	2.32		1.06		505.00	10.97	95.48	3.73	3.18		
70.00	3.35	0.64			1.04		525.00	11.07	97.06	3.77	3.03		
80.00	3.72	0.59	2.31		1.28		545.00	11.16	89.19	3.79	3.08		11,160 ± 120
130.00	5.58	1.30	2.11		1.25		559.00	11.19		3.82			
140.00	5.95	2.78			1.54		605.00	11.28	94.74		2.77		
150.00	6.32	0.00	2.30		1.35		615.00	11.30		4.38			
160.00	6.69	3.85			1.50		625.00	11.32	29.41	4.33			
170.00	7.06	2.38	2.14		1.29		645.00	11.36	16.31	3.96	3.24		
180.00	7.21	5.66					660.00	11.39		4.12			
190.00	7.35	1.30	2.12				705.00	11.49	20.00	4.19	2.97		
230.00	7.93	1.72	2.18		0.77		725.00	11.53	16.67	4.37	3.10		
240.00	8.08	1.28			0.85		745.00	11.57	62.50	4.42	3.19		
250.00	8.22	1.12	2.43		1.32		762.00	11.60	69.65		2.81		
260.00	8.37	2.33			1.20		763.00	11.60		4.45			
270.00	8.52	0.00			1.61		825.00	11.73	60.00	4.52	3.20		11,730 ± 170
280.00	8.66	0.00			1.63		845.00	11.77	6.67	4.39	2.56		
290.00	8.81	3.57	2.75		1.57		862.00	11.81		4.24			
335.00	9.46		2.91				904.50	11.89	12.90	4.25	2.51		
347.00	9.64	10.53			1.92		925.00	11.93	11.11	4.33			
351.00	9.69	14.47		2.28			945.00	11.98	9.28	4.21	2.71	2.34	
355.00	9.75	9.09					959.00	12.00		4.10			
359.00	9.81	12.73			1.66		1025.00	12.14	8.74	3.99	2.49		
361.00	9.84	26.09		2.26			1099.50	12.29	26.99	3.98	2.81		
363.00	9.87	30.43					1158.00	12.41	8.64	3.85	2.72		12,410 ± 150
365.00	9.90	20.37		2.02			1355.00	12.53	12.28	4.09			
367.00	9.93	17.31					1390.00	12.55	10.81	4.27			
369.00	9.96	21.05		2.39			1449.00	12.58	8.51	4.32			
375.00	10.04		3.02				1454.00	12.59			2.76		12,585 ± 150
381.00	10.13	30.33					1459.00	12.62	17.78				
383.00	10.16	16.67			1.92		1505.00	12.66	5.55				
385.00	10.19	6.80			1.88		1515.00	12.67		4.29			
387.00	10.22	7.69			1.79		1537.50	12.70			2.90		
389.00	10.25	9.28					1560.00	12.74	13.64	4.30			
391.00	10.28	17.78			1.81		1722.00	12.97	10.88	4.18			
393.00	10.31	13.79					1763.00	13.02	5.55				
394.00	10.32				1.96		1794.00	13.07	5.26				
395.00	10.34	10.77					1857.00	13.16	37.50	3.88			
397.00	10.36	16.67					2023.00	13.39	91.20	3.33	2.73		
399.00	10.39	19.44		2.53			2060.00	13.45	98.00	3.43	2.45		13,445 ± 155
405.00	10.48	33.33	3.66, 3.87				2094.00	13.65	96.67	3.42	2.71		
407.00						10,510 ± 115	2163.00	14.07	97.46	3.38	2.33		
411.00	10.53	83.33					2194.00	14.26	94.74	3.38	2.15		
415.00	10.55	76.47					2262.00	14.68	95.83		2.39		
419.00	10.57	88.46		2.99			2263.00	14.68		3.64			14,685 ± 120

Samples from 399 cm and above are 2 cm thick, and 10 cm thick below. The values in ref. 15 have been corrected by -30 cm for depths ≥ 429 cm to account for missing sediment. $\delta^{18}\text{O} = [(^{18}\text{O}/^{16}\text{O})_{\text{sample}} / (^{18}\text{O}/^{16}\text{O})_{\text{PDB}}] - 1$.

* Ages are calculated by interpolation between AMS-dated levels.

† AMS ^{14}C ages are corrected for surface ocean ^{14}C activity by -440 yrs. Accession numbers are given in ref. 15.

this estimate has been compromised by the combined effects of a relatively low sedimentation rate, bioturbation and changing faunal abundances.

To overcome these limitations, we measured faunal changes in core Troll 3.1 from the Norwegian Trench, where average deposition rates during the deglaciation are 5 m kyr^{-1} (ref. 15). These rates are 20–50 times higher than in previously studied cores from the open North Atlantic, and much too high to be significantly affected by bioturbation. The core site is located in the northern North Sea, an area where warm, salty Atlantic water bleeding off the main axis of inflow to the Norwegian Sea strongly influences local salinity and SST. Instrumental records show that salinity and SST variability in the area parallel changes in those properties throughout the entire North Atlantic^{16,17}. Core Troll 3.1 is thus ideally placed to monitor changes in the flux of Atlantic waters entering the Norwegian Sea basin and, by inference, water-mass changes and frontal movements in the open North Atlantic.

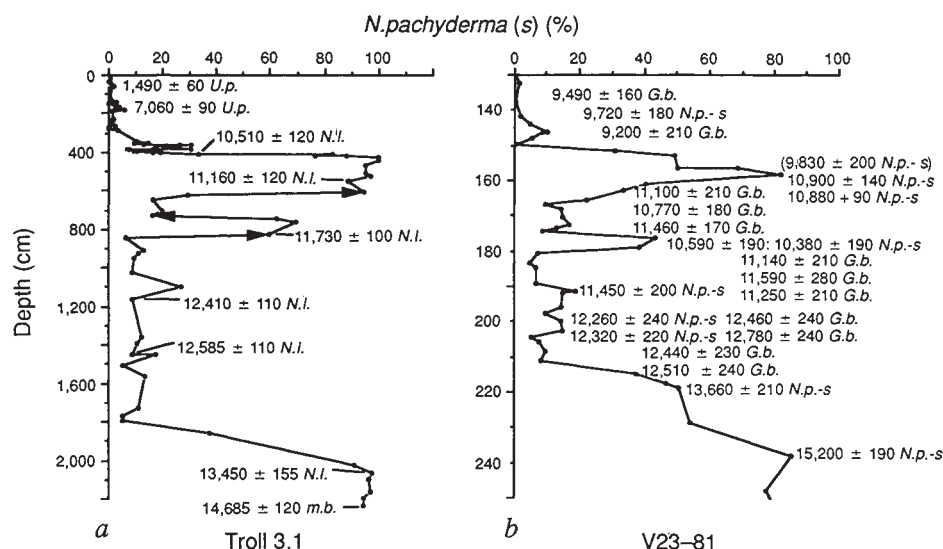
Figure 2 shows the percentage of *N. pachyderma* (*s*) plotted against core depth for Troll 3.1, with reference to a previously dated record for core V23-81 (Fig. 1), located west of Ireland. In both records at least three brief but distinct increases in polar fauna punctuate the deglaciation, with the Younger Dryas

increase (~ 11 – 10 kyr BP) being the most prominent in both. The striking correlation between the two records, each located within the present trajectory of the North Atlantic Drift, confirms that both records track ocean-wide changes in circulation. The advantage of the Troll 3.1 record is that deposition rates are high enough to permit measurement of the true amplitudes and rates of faunal changes during the deglaciation. Deglacial deposition rates in V23-81 are only 15 – 25 cm kyr^{-1} and ^{14}C dates show several stratigraphic inversions¹⁸. In the following discussion we therefore rely on the timing of faunal changes deduced from ^{14}C dates in Troll 3.1 (Fig. 2a and Table 1).

Deglacial circulation change

According to faunal changes observed in Troll 3.1, Atlantic water began to enter the Norwegian Sea 13.5 kyr BP , and near-interglacial levels of inflow were established by $\sim 13.1 \text{ kyr}$ ($1,800 \text{ cm}$), persisting until 11.7 kyr BP . A series of small, brief reductions in inflow occurred between 12.6 and 12.4 kyr BP . Subsequent transitions between high and low inflow regimes were much larger, often spanning less than 40 yr of sedimentation (as noted by the arrows in Fig. 2a). The first of the major decreases in inflow occurred at 11.7 kyr BP and was followed by an abrupt increase of similar magnitude ~ 200 years later.

FIG. 2 a, Percentage of *N. pachyderma* (s) against depth for core Troll 3.1 (60°46.7' N, 3°42.8' E, 332 m water depth) in the Norwegian Trench. Included are AMS ^{14}C dates on associated monospecific samples of benthic foraminifera (U.p. = *Uvigerina peregrina*, N.I. = *Nonion labradoricum*, m.b. = mixed benthics) from ref. 15. Despite the large difference in depth scales, the down-core pattern in a is similar to that in core V23-81 (from ref. 10) b, located off Ireland. AMS dates on *N. pachyderma* (s) (N.p.-s) and the subpolar species *Globigerina bulloides* (G.b.) for V23-81 are from refs 18 and 51. G.b. dates bracketing the N.p.-s peak at 175 cm are compatible with the age of a similar peak in Troll 3.1. The Younger Dryas is marked by the largest N.p.-s peak in each core and also by an independently dated ash horizon in V23-81 (refs 10 and 52). Rates of SST change implied by faunal variations between intervals marked by arrows equal or exceed 5°C in 40 yr or less.



N. pachyderma (s) levels then rose again at 11.2 kyr BP, indicating decreased inflow at the start of the Younger Dryas cold period. Ocean cooling during the Younger Dryas ended at 10.5 kyr BP, with the resumption of surface inflow from the Atlantic occurring in less than 100 yr. The apparently slower faunal change during this period is probably an artefact of the decreasing sedimentation rate, which drops to $\sim 1 \text{ m kyr}^{-1}$, rather than a real increase in ocean response time. The restored circulation at the end of the cold part of the Younger Dryas lasted only a few hundred years, and was followed by another reduction in circulation beginning at ~ 9.7 kyr BP (385 cm), also lasting a few hundred years, before the establishment of a stable interglacial circulation regime by ~ 8.8 kyr BP (290 cm).

As *N. pachyderma* (s) are abundant only in polar and subpolar surface waters, the Troll data indicate that the southeast Norwegian Sea was alternately influenced by cold and temperate water masses, reflecting sudden changes in the poleward advection of warm subtropical surface waters. Cold oscillations persisted for periods of 200–700 ^{14}C years and transitions between cold and warm states were extremely rapid, taking no more than 40 yr. An estimate of the magnitude of SST variations implied by faunal changes in Troll 3.1 is possible because the distribution of *N. pachyderma* (s) with respect to present SSTs is well documented. This species lives only in waters colder than $\sim 10^\circ\text{C}$ and constitutes more than 95% of the fauna at summer temperatures below $\sim 5^\circ\text{C}$ (refs 11 and 19), thus providing a reliable indicator of temperature across a range of $\sim 5^\circ\text{C}$. Rates of faunal change before and at the start of the Younger Dryas therefore correspond to summer SST changes of $\geq 5^\circ\text{C}$ over less than 40 yr. This is commensurate with the rates of change implied by ice-core data².

Surface circulation and ice-sheet $\delta^{18}\text{O}$

It has been hypothesized that air temperatures in the circum-Atlantic region are governed by sudden changes in the rate of thermohaline overturn in the Atlantic³, suggesting that the history of ice-core $\delta^{18}\text{O}$ and Atlantic faunal variations should be similar. Figure 3a shows $\delta^{18}\text{O}$ from the upper part of Dye 3, the highest-resolution core obtained from the Greenland ice sheet. A tentative age model for the upper part of the core is afforded by comparison with dated $\delta^{18}\text{O}$ variations in marls from Lake Gerzensee, Switzerland^{3,20} (Fig. 3a). Major isotopic features of the Dye 3 record are shared by two other long cores from different locations on Greenland^{1,21} (Fig. 1) indicating that they reflect climate shifts affecting the whole ice sheet. The Dye 3 record is compared in Fig. 3b with the *N. pachyderma* (s)

data from Troll 3.1, plotted against age. The excellent correlation is illustrated in Fig. 3c, with the $\delta^{18}\text{O}$ measured in the ice sheet closely matching the evidence for changing SST in the North Atlantic between 14 and 10 kyr BP. Thus rapid changes in poleward flux of ocean heat are shown to have governed air temperatures in the region^{3,4}. If the ice-core $\delta^{18}\text{O}$ variations were dominated instead by changes in the location, temperature and isotopic composition of source waters in the vicinity of the polar front²², contemporaneous isotopic shifts should have been more pronounced nearer the coast, opposite to that observed at Renland²¹. Rather, deuterium-excess data for Dye 3 indicate that vapour reaching high elevations in the Arctic originated primarily at subtropical latitudes²³, well south of the area directly affected by polar front movements.

After ~ 10 kyr BP, precipitation rates over the ice sheet increased considerably²⁴, distorting the graphical correspondence in Fig. 3 between depth-related features in Dye 3 and age-related features in Troll 3.1. But annual layer counting in Dye 3 shows the age of the point at which $\delta^{18}\text{O}$ values stabilize at Holocene values to be $\sim 9,800$ calendar yr BP²⁴, corresponding to a ^{14}C age of $\sim 8,800$ yr BP²⁵. This is in good agreement with the interpolated ^{14}C age for the level at which the proportion of *N. pachyderma* (s) remains at Holocene values, indicating that the correlation between the ocean and ice-core records can be extended into the Holocene.

SSTs and European climate

Numerical climate models suggest that changing SSTs in the North Atlantic control surface air temperatures both locally and downwind over parts of Europe and Asia⁹. Similarities between meteoric $\delta^{18}\text{O}$ records at both Dye 3 and Lake Gerzensee, and the faunal variations in Troll 3.1, show that ocean-modulated changes in air temperature spread downstream of the Atlantic at least as far as central Europe. In the British Isles, where the oceanic climate signal should have been especially pronounced, a composite record of temperature changes during the deglaciation based on ^{14}C -dated beetle remains²⁶ is in good agreement with the Troll data. In particular, stepwise coolings at ~ 12.5 and ~ 11.8 kyr BP and a brief warming ~ 11.3 kyr just before the Younger Dryas cold period can now be attributed to changes in Atlantic SST.

The new ocean data call for revision of the existing late-glacial climato- and chrono-stratigraphy for northwest Europe and Scandinavia. Whereas the standard chronology²⁷ indicates only a single cold episode before the Younger Dryas, faunal data in Troll 3.1 indicate that there were two such coolings. Evidence

of two coolings is also present in the classical late-glacial pollen zonation from Denmark, one before and another within the Allerød warm period²⁸. Additional evidence for multiple coolings during this interval is available from sites in southern Norway^{29,30} and southwest Sweden³¹. We associate the first of these coolings with the 'Older Dryas', as it represents the most distinctive climatic reversal in terrestrial records prior to Allerød warming. The events are correlated with evidence of reduced Atlantic water inflow dated ~12.5 kyr BP (Fig. 4), which may have led to the well documented ice-sheet advance in Western Norway that culminated ~12.2 kyr BP³². We equate evidence for the second cooling with the strong reduction of warm water inflow beginning 11.7 kyr BP and refer to this as the intra-Allerød cold period (IACP) in accordance with classical Danish pollen stratigraphy²⁸.

Previously accepted boundary ages of 12.0 and 11.8 kyr BP for the Older Dryas chronozone²⁷ were based on an averaging of the most reliable dates associated with evidence for cooling in lake sediments. In all likelihood, this reflects a mixture of IACP and Older Dryas events, and may explain why evidence for cooling around the Older Dryas period has been difficult to correlate throughout northwest Europe and the British Isles.

Troll 3.1 shows that severe ocean cooling during the Younger Dryas ended ~10.5 kyr BP, in accordance with evidence of warming and rapid glacier recession beginning at that time in southern Norway^{33,34}, Sweden³¹ and the British Isles²⁶. A notable exception to this trend are ¹⁴C dates from Ballybetagh, Ireland, suggesting that Younger Dryas cooling occurred there between 10.6–10.0 kyr BP³⁵. As climate in Ireland should be strongly influenced by the surrounding SSTs, we find these dates difficult to reconcile with the record in Troll 3.1. Following the sudden warming at the close of the Younger Dryas, a final brief reduction in Atlantic water inflow occurred ~9.7 kyr BP. We note that there is new evidence from the Jostedalsgreen region of western Norway for glacier advance and cooler temperatures at this time³⁶.

Thermohaline circulation

At present, a combination of cold air and restricted sea-ice

cover promotes deep-water formation in the Norwegian Sea and drives a compensatory northward flow of warm Atlantic surface waters into the basin. Deep waters formed in the Norwegian Sea overflow Denmark Strait into the deep Atlantic and comprise the densest and therefore deepest component of NADW (namely, LNADW)³⁷. Because of the large temperature depression associated with this watermass conversion, the formation of LNADW involves ~40% more heat release to the atmosphere per unit flux than does formation of the less dense components of NADW^{38–40}. The history of LNADW should therefore play a pre-eminent part in North Atlantic climate change, and be intimately tied to surface conditions within the Norwegian Sea.

Figure 4 compares evidence for changes in surface circulation at Troll 3.1 with geochemical evidence for abyssal circulation change (the Cd/Ca ratio in benthic foraminifera) in core EN120-GGC1, which lies within the flow path of LNADW at a depth of 4.5 km in the western Atlantic^{41,42}. After 13.5 kyr BP when warm Atlantic surface water first entered the Norwegian Sea basin, the flow of LNADW was restricted during the periods when the Norwegian Sea was cold (Fig. 4). In particular, both surface and abyssal circulation are shown to have been mostly suppressed from the start of the Younger Dryas until the middle of the Preboreal^{41,42}, eliminating any lingering doubt⁴³ that formation of the densest components of NADW was interrupted during the Younger Dryas. Correlation of the evidence for cooling during the Older Dryas and IACP with intervals of high Cd/Ca ratios (reduced LNADW) in EN120-GGC1 is less certain owing to the poorer dating during this interval. Recent evidence from the deep Southern Ocean for deglacial changes in the $\delta^{13}\text{C}$ of Circumpolar Deep Water (CPDW) provides an additional monitor of changes in the net flow of NADW, but does not differentiate its individual components. This shows that brief decreases in NADW flow occurred at ~12.7 kyr and ~11.7 kyr BP, following an initial increase between ~13.5 and ~13.1 kyr BP⁴⁴. Within dating uncertainties of a few hundred years, the Southern Ocean record indicates that overall production of NADW was reduced during the Older Dryas and IACP as suggested by EN120-GGC1 and Troll 3.1. The Southern Ocean

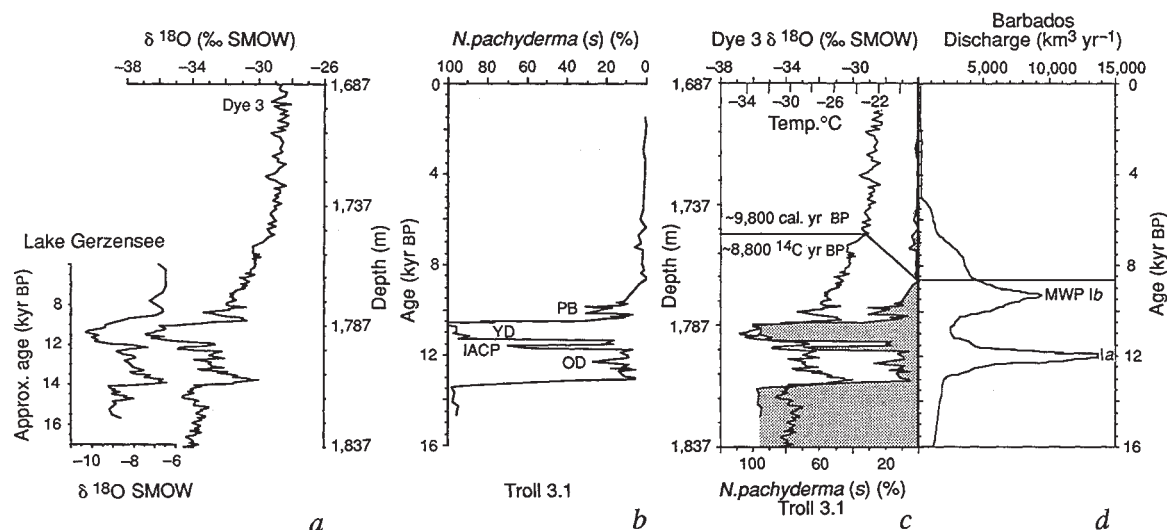


FIG. 3 *a*, $\delta^{18}\text{O}$ of the upper part of the Dye 3 ice core from South Greenland (from ref. 3) is shown with reference to dated $\delta^{18}\text{O}$ variations in marls from Lake Gerzensee, Switzerland (from ref. 20). $\delta^{18}\text{O} = [({}^{18}\text{O}/{}^{16}\text{O})_{\text{sample}} / ({}^{18}\text{O}/{}^{16}\text{O})_{\text{SMOW}}] - 1$. The linear depth scale has been chosen to maximize correlation between major isotopic shifts in the ice core record and shifts in *N. pachyderma* (s) for Troll 3.1, *b*, plotted to age using dates in Fig. 1*a*. *c*, Ocean and ice core data are overlain with the $\delta^{18}\text{O}$ -temperature scale for the ice core shown². The strong correlation permits the ¹⁴C chronology for Troll 3.1 to be transferred to the ice-core record for the

period between ~14 and 9.5 kyr. Using this timescale, it is evident that periods of accelerated global ice-sheet melting shown in *d* (from ref. 46) correspond to periods of warming as indicated by both the ice core and faunal data. A correlation line relates the ~8,800 ¹⁴C yr BP level in each record, by which time deposition rates on the ice-sheet increased due to a Holocene increase in moisture flux²⁴. Faunal shifts in Troll 3.1 are labelled to reflect proposed correlations with terrestrial evidence for cooling in northwest Europe, as discussed in the text.

record, however, lacks evidence of a significant reduction in NADW during the Younger Dryas. As there are clear indications from other locations for reduced surface flow and elimination of LNADW at that time, it is possible that CPDW was being fed by an enhanced flux of NADW at shallower depth, leading to relatively high $\delta^{13}\text{C}$ values. A gradual deglacial deepening of the mixing front between nutrient-rich, southern-source waters and the overlying, nutrient-depleted, northern-source waters is suggested by the overall trend of the decreasing Cd/Ca ratio in EN120-GGC1 (Fig. 4a). This may be viewed as evidence for an increase in overall production and density of NADW during the deglaciation, as might be expected in response to decreasing ice-sheet size and increasing insolation between 18 and 9 kyr BP. Superimposed on this Cd/Ca trend are several major oscillations, reflecting modulation of LNADW flow which we find to be correlated with changing conditions in the Norwegian Sea. By the start of the Younger Dryas period, the overall flow of NADW was apparently great enough to influence the composition of CPDW, as expected because of NADW deepening, but the temporary absence of LNADW led to extreme cooling at the surface of the North Atlantic.

Mechanisms of rapid circulation change

Broecker and colleagues^{8,45} suggest that circulation may oscillate between 'on' and 'off' modes through the effect of changing ocean heat flux on the melting rate of ice sheets situated near areas of convection. In their model, periods of enhanced thermohaline flow lead to the accelerated melting of the Fennoscandian ice sheet (FIS), which in turn damps the oceanic circulation as a result of the increase in meltwater flux. Cooling arising from the reduction in the circulation then halts the melting,

allowing salt to build up to levels compatible with renewed deep-water formation. Troll 3.1 is ideally placed to monitor such interactions, because ^{18}O -depleted meltwaters emanating from the FIS, and spreading towards regions of convection, must pass over the core site. Comparing faunal and benthic $\delta^{18}\text{O}$ (ref. 15) results in Troll 3.1 (Fig. 4c), we find that the intervals of decreasing $\delta^{18}\text{O}$ (from 12.7 to 12.4 kyr and 11.5 to 11.2 kyr) immediately precede faunal evidence for reduced circulation, the more recent melting event apparently triggering the Younger Dryas. The decrease in benthic $\delta^{18}\text{O}$, beginning about 50 yr after the onset of Younger Dryas cooling, was caused by the initial discharge of stored meltwater from the Baltic Ice Lake^{15,31}, which may have prolonged and intensified suppression of the circulation during the Younger Dryas. Another interval of decreasing benthic $\delta^{18}\text{O}$ after 10.4 kyr BP is associated with the final discharge of the Baltic Ice Lake^{15,31} and with evidence for renewed ice-sheet recession in western Norway^{32,33}. Discharge and melting during this time may have triggered the subsequent reduction in circulation during the Preboreal. Clear evidence for the reduction in circulation at 11.7 kyr being triggered by meltwater is lacking in Troll 3.1. The earliest meltwater event, between 15 and 13.5 kyr BP^{15,49}, occurred before Atlantic water entered the basin and was most probably caused by increasing insolation and global sea level¹⁵.

In contrast to the sensitive feedback between circulation and FIS melting indicated by the faunal and benthic $\delta^{18}\text{O}$ data in Troll 3.1, the rate of global ice-sheet melting⁴⁶ seems to have been controlled by longer-term, millennial-scale trends in the thermohaline circulation and associated changes in air temperature (Fig. 3). This observation rests not only on ^{14}C -based correlations in Fig. 3, but is also suggested by variations of $\delta^{18}\text{O}$

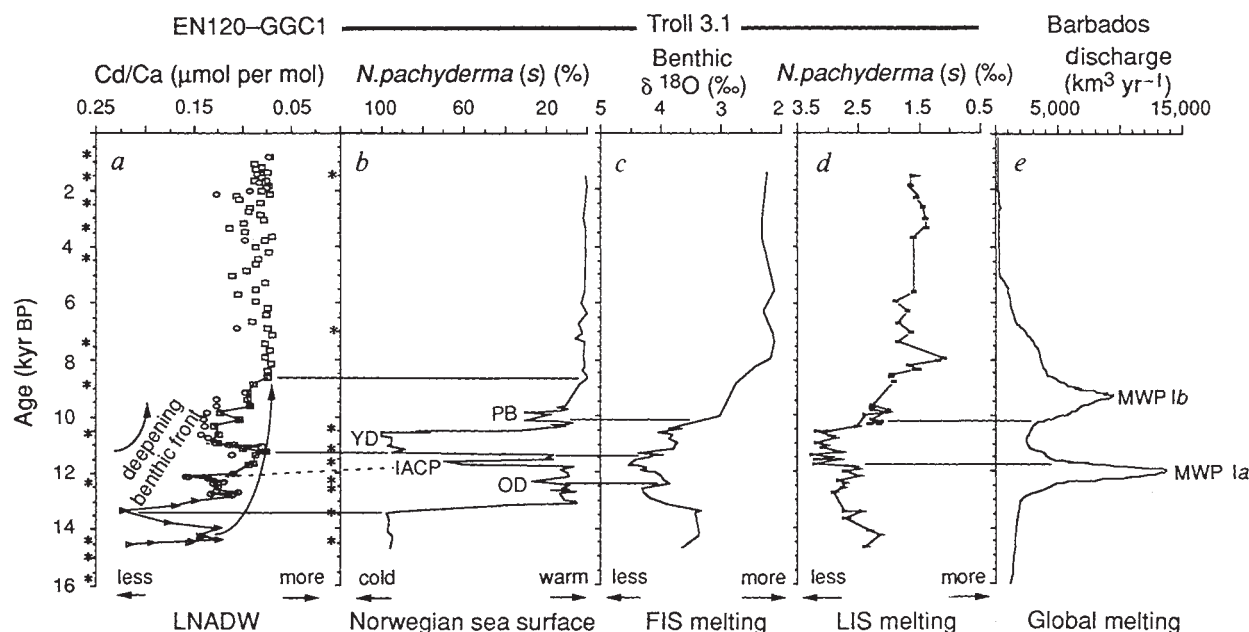


FIG. 4 Cd/Ca ratio ($\mu\text{mol per mol}$) in benthic foraminifera from Bermuda Rise core EN120-GGC1 (from ref. 41), a, correlated to Troll faunal results (b) showing that after 13.5 kyr BP, the flow of LNADW was restricted when the Norwegian Sea was cold. AMS control points for Troll 3.1 and age control points for GGC1, from ref. 42, are noted by asterisks on their respective axes. The dashed line between a and b refers to a proposed correlation in an interval where age control is relatively poor in GGC1. This correlation is supported by recent $\delta^{13}\text{C}$ evidence from the Southern Ocean (ref. 44). The monotonic decline in Cd/Ca during deglaciation is attributed to gradual deepening of the mixing front between northern and southern source waters. c, Benthic $\delta^{18}\text{O}$ in Troll 3.1, from ref. 15, mainly reflects the melting history of the Fennoscandian ice-sheet. The solid lines between b and c shows

that evidence for increased melting immediately precedes faunal evidence for cooling. The detailed phasing between isotope and faunal shifts can be clearly seen in Table 1. d, Planktonic $\delta^{18}\text{O}$ results for Troll 3.1, which reflect primarily the impact of Laurentide ice-sheet melting on the $\delta^{18}\text{O}$ of the open Atlantic, are measured using *N. pachyderma* (s) before 9.7 kyr BP, and for *N. pachyderma* (d) after 10.3 kyr BP (Table 1). The *N. pachyderma* (d) results have been corrected to *N. pachyderma* (s)-equivalent values by addition of 0.35‰, based on the overlapping samples. Correlation between d and the record of global ice-sheet melting rate, e, confirms the millennial-scale relationship between faunal shifts and global melting implied by ^{14}C -based correlations in Fig. 3.

in *N. pachyderma* from Troll 3.1 (Fig. 4 and Table 1). These variations reflect primarily changes in salinity of the open North Atlantic, controlled by melting of the Laurentide ice sheet (LIS). As the LIS is volumetrically the most important contributor to the deglacial sea-level rise, planktonic $\delta^{18}\text{O}$ from open Atlantic cores is expected to mimic the sea-level record⁴⁶. Troll 3.1 clearly shows this trend, with the most prominent decreases in planktonic $\delta^{18}\text{O}$ occurring at the times of meltwater pulses 1a and 1b⁴⁶, while higher $\delta^{18}\text{O}$ (reduced LIS melting) is associated directly with faunal evidence for restricted surface circulation (Fig. 4d, e).

It is too early to conclude with certainty that the FIS and LIS have different roles within the coupled ocean-atmosphere-ice-sheet climate system. The observation that global ice-sheet melting rates were controlled by millennial-scale trends in thermohaline circulation and circum-Atlantic air temperatures may be partly an artefact of the relatively low resolution of the deglacial sea-level record, its integration of Antarctic melting history with that of the LIS and FIS, and the distance of Troll 3.1 from actual sites of LIS discharge to the oceans. Indeed, dated planktonic $\delta^{18}\text{O}$ curves from the Gulf of Mexico^{18,47} and the subtropical Atlantic^{48,42} indicate that, after 13.5 kyr BP, LIS melting was restricted to times when, according to the Troll 3.1 data, circulation was enhanced. Thus, although the melting rate of both ice sheets may have been governed by changes in thermohaline circulation, the extent to which their individual meltwater contributions affected thermohaline flow may have been quite different. LIS meltwater must travel further to reach areas of convection in the Norwegian Sea. Consequently, greater or more prolonged LIS melting may be required for there to be an equivalent damping effect on the circulation⁶. The differing time constants and effectiveness of meltwater transport for the two

ice sheets may permit LIS meltwater to build up in the Atlantic, leading to a nonlinear response of the circulation to ice-sheet melting. This may account for the observed pattern of increasingly severe circulation shut-downs during the deglaciation, culminating in the Younger Dryas. Smoothing of these century-scale phenomena, as might be typical of lower-resolution records of melting and circulation change, would in fact yield the millennial-scale association between global ice-sheet melting, circulation and air temperatures noted earlier.

In summary, we find evidence that deglacial shifts in the poleward flow of warm Atlantic surface waters occurred extremely rapidly, typically within a 40-yr period. These controlled the air temperatures in the circum-Atlantic region, and were linked with variations in the flow of LNADW, whose formation today delivers more heat to the atmosphere than the other components of NADW. After 13.5 kyr BP, when warm Atlantic water first penetrated the Norwegian Sea, circulation and air temperatures appear to have oscillated in conjunction with the melting history of the FIS, as hypothesized by Broecker and colleagues^{8,45}. These circulation changes seem also to have governed the melting history of the LIS. We speculate that the removal of the ice-sheet margins from the high-latitude continental shelves between 15 and 13.5 kyr BP^{15,49,50} constituted a fundamental change in climate boundary conditions, and led to sudden warming of much of the North Atlantic (L.D.K. and S.J.L., manuscript in preparation). This change may have permitted the formation of deep water in the Norwegian Sea, drawing Atlantic water into the basin for the first time. This set the stage for the pronounced deglacial oscillations that we document after 13.5 kyr, and led ultimately to the establishment of today's stable interglacial circulation regime. □

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Cloning of cDNAs for Fanconi's anaemia by functional complementation

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Fanconi's anaemia is a rare autosomal recessive disorder characterized by progressive pancytopenia and a cellular hypersensitivity to DNA crosslinking agents. Four genetic complementation groups have been identified so far, and here we use a functional complementation method to clone complementary DNAs that correct the defect of group C cells. The cDNAs encode alternatively processed transcripts of a new gene, designated *FACC*, which is mutated in group C patients. The predicted *FACC* polypeptide does not contain any motifs common to other proteins and so represents a new gene involved in the cellular response to DNA damage.

FANCONI'S anaemia, xeroderma pigmentosum and ataxia telangiectasia are all representatives of a group of human genetic disorders characterized by hypersensitivity to DNA-damaging agents and an increased propensity to the development of malignancy. On this basis they have been tentatively classified as DNA-repair disorders¹. As a member of this group of diseases, Fanconi's anaemia is particularly interesting because, in addition to the hypersensitivity to damage of their DNA, patients have skeletal abnormalities, notably of the thumb and radius, progressive bone marrow failure, and an increased incidence of acute myeloid leukaemia^{2,3}. These defects may result from the failure of a developmentally important function⁴. Cells from patients with Fanconi's anaemia have a high level of spontaneous chromosomal aberrations compared with cells from unaffected individuals⁵. This phenotype is more pronounced when DNA-crosslinking agents such as mitomycin C or diepoxybutane are used to induce chromosome damage^{6,7}, and is used to confirm a clinical diagnosis of Fanconi's anaemia.

It has been difficult to identify a biochemical defect even knowing the physical and cellular features of Fanconi's anaemia. Two hypotheses are at present under investigation. One is that the cell cannot repair damaged DNA as the defective protein is directly involved in recognizing, modifying or repairing crosslinks⁸. The other is that the cell is unable to respond to the oxidative stress caused by DNA-crosslinking agents because of a defect in one of the detoxification mechanisms that remove free radicals or oxygen byproducts⁹. Given that at least four complementation groups have been identified in this anaemia³⁵, it is possible that both explanations are valid, although confirmation must await elucidation of the basic defects. We report here a first step towards this goal with the cloning of cDNAs from complementation group C (FA(C)).

Cloning of the *FACC* cDNA

As the cellular defect in Fanconi's anaemia is fully complemented in somatic cell hybrids^{10,35}, we considered that functional complementation of cellular mitomycin C and diepoxybutane hypersensitivity using a transfected cDNA expression library would be a viable way to clone cDNAs defective in this anaemia. An Epstein-Barr virus (EBV)-based cDNA expression shuttle vector, pREP4, was used to construct the library (Fig. 1a)¹¹. Such vectors are maintained episomally¹², and the plasmids conferring resistance can easily be recovered through shuttling back to *Escherichia coli*. This cloning strategy both enhances the transfection efficiency of the library into human cells and eliminates the need to reclon the complementing cDNA¹³.

In the complementation experiments, an FA(C) cell line, HSC536N, was chosen as a recipient for the cDNA library because of its extreme sensitivity to mitomycin C and diepoxybutane¹⁴. Following selection, episomal DNA from the surviving cells of each independent pool was isolated and characterized (Table 1). Many of the plasmids recovered from the selected cells were merely passengers and did not confer resistance to either mitomycin C or diepoxybutane, because the EBV replicon in the pREP4 cloning vector is highly efficient and plasmids may be maintained in lymphoblasts even in the absence of direct selection¹⁵. Passengers and complementing cDNAs were distinguished from each other because three independent pools of cells had been maintained during the selection. Only plasmids present at elevated levels in one pool and/or represented in more than one pool were considered to encode the candidate *FACC* (for FA group C complementing) cDNAs. Eight candidates were identified after restriction mapping 216 plasmids recovered from the selected cells (Table 1). To determine which of these conferred resistance to mitomycin C and/or diepoxybutane, representative plasmids were transfected into HSC536N cells. Sensitivity to both reagents was corrected to normal levels with only three of the eight candidates, designated pFAC3, pFAC8 and pFAC4 (Fig. 2, and Tables 1 and 2a).

Detailed restriction mapping of pFAC3, pFAC8 and pFAC4 revealed that they contain 4.6, 3.2 and 2.3 kilobase-pair (kb) cDNA inserts, respectively (Fig. 1b). The cDNAs overlapped in sequence, and subsequent analysis confirmed that they represent alternatively processed transcripts of the same gene (see below). To demonstrate further that the three plasmids specifically complement the FA(C) defect and do not merely confer nonspecific resistance to mitomycin C and diepoxybutane, each was transfected into lymphoblast lines representative of the other Fanconi's anaemia complementation groups. Full correction of the defect was manifested only in HSC536N (Table 2b), leading to the conclusion that the three plasmids confer specific complementation and are the *FACC* cDNA.

Characteristics of *FACC* cDNA

The entire sequence of the *FACC* cDNA and its putative translation product are presented in Fig. 1c. The cDNA is 4,566 base pairs (bp) in length, and contains an open reading frame of 1,674 bp encoding a predicted protein of 557 amino-acid

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residues starting at base 256. Although this is the first in-frame ATG with a good consensus ribosome-binding site¹⁶, several other downstream in-frame ATG codons, if they are used, would yield polypeptides starting at residues 16, 48 or 55 of the indicated FACC polypeptide.

Alternatively processed forms of the cDNA are encoded on pFAC3, pFAC4 and pFAC8 (Figs 1b and c). Two different 5' untranslated regions (UTRs) were identified, converging 77 bases upstream of the initiation codon. Sequence analysis does not reveal any conserved splice acceptor or donor sites surrounding this location¹⁷, suggesting that the two different 5' UTRs are not artefacts of cDNA synthesis attributable to the presence of unprocessed introns. Rather, the different 5' UTRs probably represent alternatively spliced exons, and these are tentatively identified in Fig. 1c as exon 1 (as found on pFAC4 and pFAC8) and exon 1A (as found on pFAC3). To probe the extent of heterogeneity within each exon, the 5' UTR sequence of 24 clones picked at random from those recovered from each independently selected pool of HSC536N cells was determined (Table 1). Five clones contained exon 1A and all five originated at the same base. The remainder of the clones contained exon 1 and were heterogeneous in length, with the different 5' ends indicated by asterisks in Fig. 1c.

The 3' UTRs of each cDNA also differ in length and contain identical sequences which are truncated at different points to generate the 2.3, 3.2 and 4.6 kb cDNAs (Fig. 1b, c). Northern blot analysis (Fig. 3) clearly detects three messenger RNAs of this size in lymphoblasts, and sequence analysis does not reveal an extensive internal poly(A) tracts that would facilitate misprimed cDNA synthesis, confirming that the different cDNAs represent actual transcripts of the *FACC* gene and are not artefacts of library construction. The longest 3' UTR has a perfect consensus polyadenylation signal¹⁸ at base 4,545, whereas the two shorter 3' UTRs have only poor matches, suggesting that the size differences are the result of transcriptional read-through of the first two polyadenylation signals rather than alternative splicing. Interestingly, the longest transcript also seems to be the most abundant (Fig. 3) and contains a series of direct 35-bp repeats preceded by a 12-bp palindrome starting at base 3,356 (Fig. 1c).

The variations among the *FACC* transcripts as described are confined entirely to untranslated regions, with no differences detected throughout the coding sequences for each of the cDNAs examined. If the predictions from the cDNA sequence are correct, the *FACC* protein has an M_r of ~63,000 and contains a preponderance of hydrophobic amino acids (average hydrophobicity, 0.17; ref. 19), although there are no identifiable transmembrane domains²⁰. No significant homologies were found between the *FACC* cDNA or its predicted amino-acid sequence and data base sequences (Fig. 1c); *FACC* is therefore a new gene involved in the cellular response to DNA damage.

Mutations in *FACC*

Confirmation that aberrant expression of *FACC* causes the defect in FA(C) patients was obtained by analysing the coding region of *FACC* cDNA for the presence of mutations after amplification by polymerase chain reaction (PCR) of reverse-transcribed RNA (ref. 21). HSC536N cells, which represent the only confirmed FA(C) cell line, have a T-to-C transition at base 1,913 that changes codon 553 from leucine to proline (L553P) (Fig. 4). Because L553P leads to the loss of a *BbvI* restriction enzyme digestion site, we were able to determine that the patient is heterozygous for L553P and that this mutation is maternally inherited (data not shown). The inherited paternal mutation must therefore lead to a non-expressed allele.

No sequence polymorphisms were detected in the *FACC* coding region of two normal and five FA cell lines (not group C) that constitute the other three Fanconi complementation groups. But in two out of four unclassified FA cell lines a deletion of a single G at base 322 in one allele was detected

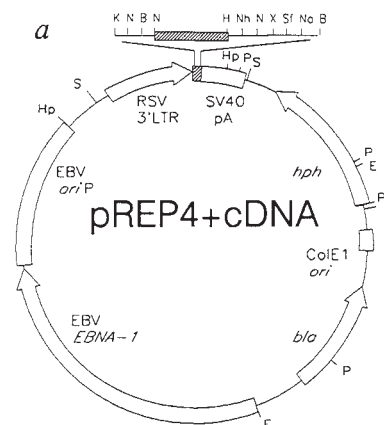


FIG. 1 a, Restriction map of the pREP4 EBV-shuttle vector¹¹ used to construct the cDNA expression library. The open boxes indicate the orientation of the EBV origin of replication (*oriP*) and nuclear antigen (*EBNA-1*), the hygromycin (*hph*)- and ampicillin-resistance genes (*bla*), and the bacterial origin of replication (*ColE1 ori*) required for selection and replication, as well as the Rous sarcoma virus (RSV)-3' long terminal repeat (LTR) and simian virus 40 (SV40) polyadenylation signal used to drive cDNA expression. The hatched box indicates the cDNA cloning site. b, Restriction maps of the insert cDNAs from the indicated plasmids. The open box indicates the location of the common open reading frame, the closed box indicates common flanking sequences, and the hatched box indicates the alternatively spliced exon 1A. c, Nucleotide sequence of the *FACC* cDNA. The cDNA is numbered from the first base of exon 1, with the sequence of exon 1A aligned with respect to the common splice junction at base 179. The amino-acid sequence of the predicted protein is indicated in single-letter code. The different 5' transcript start sites in exon 1 are indicated by asterisks. The alternative polyadenylation signals, 12-bp palindrome, and 35-bp direct repeats in the 3' UTR are indicated by the open boxes and open arrows, respectively. Abbreviations: B, *Bam*HI; E, *Eco*RI; Ev, *Eco*RV; H, *Hind*III; Hp, *Hpa*I; K, *Kpn*I; N, *Not*I; Na, *Nae*I; Nh, *Nhe*I; P, *Pst*I; S, *Sal*I; Sf, *Sfi*I; Sm, *Sma*I; X, *Xho*I; Xb, *Xba*I.

METHODS. The cDNA library was constructed in pREP4 using the Moloney murine leukaemia virus-RNaseH⁻ reverse transcriptase (BRL) in conjunction with vector-primed synthesis to enhance the yield of full-length inserts oriented with respect to the RSV-3'LTR promoter and SV40 polyadenylation signal. To prepare the vector, 20 μ g of a phosphorylated *Hind*III/poly(T) oligonucleotide primer (AGCT(T)₅₀) was ligated to 50 μ g *Hind*III-digested pREP4. The vector was digested with *Pvu*II to generate a 5' blunt end and then purified by chromatography, first over Sephacryl S-200 (Pharmacia) to remove unreacted primers as well as the short *Pvu*II-primer fragment, and then over oligo(dA) cellulose to purify the poly(T)-tailed vector. Poly(A)⁺ RNA for the library was isolated through two rounds of oligo(dT) cellulose chromatography from HSC93 lymphoblast cells which were grown in medium containing a sublethal dose of 500 nM diepoxybutane (DEB)³¹. To prime cDNA synthesis, 5 μ g tailed vector was annealed with 1 μ g RNA, and first and second strand cDNA synthesis was performed using standard methods³². The cDNA was blunt-ended with T4 DNA polymerase, and hemi-phosphorylated *Bam*HI-*Not*I adaptors (Pharmacia) were ligated onto the ends, phosphorylated, and the completed cDNA/vector recircularized. An aliquot of the ligation mixture was electroporated into *E. coli* DH10B, and the resulting library amplified in semisolid agarose to minimize skewed representation of clones³³. The library is derived from 5×10^6 independent CFU, with an average insert size of ~1 kb. To sequence individual cDNAs, the inserts from each plasmid were first subcloned in their entirety into pBluescript as *Not*I or *Bam*HI fragments. Both strands of the coding region were sequenced either as further subclones using internal restriction enzyme sites or using *FACC*-specific oligonucleotide primers. The cDNA sequence and the translated protein were tested for homology to sequences in the GenBank (release 70) or EMBL (release 25) data bases and their translated counterparts. No significant homologies were detected. Further, a search through the NBRF-PIR (release 29), Swiss-Prot (release 17) and EMBL-Prosite (release 6.0) data bases using the predicted amino-acid sequence did not uncover homologies or reveal functional motifs.

Exon 1
1 ACTGCTGACACGTGTGCGCGCGCGGCTCCACTGCCGCGGCGACCGCGGAAATTCACAAAAAATCAAAAGGCAATACGAGGCCAAGCCAAATTTTCAAGCCACAGATCCCGGGCGG
Exon 1A GAGCCCCGGAGAGCGGGAGCGGTGTGGCGCTTTGG

121 TGGCTTCTTTCCGCCACTGCCAACTGCTGAAGCAGCTCCCGCGGAGGACCCCGATTATGTGTGCCGACCATTTCTTCAGTGCTGACAGGCTGCTGTGAAGGACATCACCTT
TTCTTTTGTTCATTGACGCCAGGCAGCTATGTCTCTTCAAAGGAGAGGAGCAAGC

241 TTCGCTTTTCCAAGATCGGCTCAAGTATCAGTAGCTTTCTGTGATTACGTTTTGGATGCGAAGCTTCTGTATGGGATCAGGCTTCCACTTTGGAAACCCAGCAAGACACCTGT
M A Q D S V D L S C D Y O F W M Q K L S V W D O A S T L E T O Q D T C 35

361 CTTACAGTGGCTCAGTTCAGGAGTTCCTAAGGAAGATGTATGAAGCCTTGAAGAGATGGATTCTAATACAGTCATTGAAAGATTCCCCACAATTGGTCAACTGTTGGCAAAAGCTTGT
L H V A Q O F O E F L R K M Y E A L K E M D S N T V I E R F P T I G Q L L A K A C 75

481 TGGAACTCTTTATTTAGCATATGATGAAGCCAAAAATCTAATATGGTGCTTATGTCTAATTAACAAGAACCAGAGAAATCTGGACATCAAACTCTGGATACAG
W N P F I L A Y D E S O K I L W C L C C L I N K E P O N S G O S K L N S W I O 115

601 GGTGTATTATCATATACTTTACGACTCAGATTGTATGAAGAAGTGTCTTTCTACTCAAGGCTTGGGTATGCACCTATAGATTACTCTCGTGTGCTTAAAAATATGGTTTAA
G V L S H I L S A L R F D K E V A L F T Q G L G Y A P I D Y Y P G L L K N M V L 155

721 TCATTACGCTGAACCTCAGAGAAATCATCTTAATGATTAACTCAAGGCAATGGCTCCGAGGCGAGTGGCGTCCCTGTCAAGGTTTGTGTTCCACTTATACCTGACAGAT
S L A S E L R E N H L N G F N T O R R M A E S G V P V T S L C S T Y Y L T D 195

841 GTTGACCCCTGGTGGAGGCTCTCTCATCTGTCTGACGCTGAACCTCAGGAAATCTCCAGCCAGAGTCTTTGAGGCTGTAAACGAGGCGATTTTGTGAAGAAGATTCTCTCCCG
V D P L V E A L L L I C H G R E P O E I L Q P E F F E A V N E A I L L K K I S L P 235

961 ATGTCAGCTGATCTGCCTCTGGCTCTCCGACCTTCCGACCTTGAAGCAATGCTGCATCTTTTGAAGCAATCTCACTGAGAGAAATGTCTGAGAAGGATCGAATGCTTT
M S A V V C L W L R H L P S L E K A M L H L F E K L I S S E R N C L R R I E C F 275

1081 ATAAAAGATTATCGCTGCCTCAAGCAGCTGCCACCTGCCATATCCGGTGTGTGATGAGATGTTCAAGTGTGCACCTCTGGAACCGATGGGCGCTGGAAATCATAGCCACTATT
I K D S S L P O A A C H P A I F R V V D E M F R C A L L E T D G A L E I I A T I 315

1201 CAGGTGTTTACGAGTGTCTTGAAGAGCTCTGGAGAAAGCAAGCAGGACGCGGTTTGCACCTCAAGCACTTCTTCTTACACTTCCATCTTCCGATGCTGCTGCGAAGC
Q V F T Q C F V E A L E K S K O L R F L K T Y F A C T S P S L A M V L Q D 355

1321 CCTCAAGATATCCTCGGGGACACTGGCTCAGACACTGAAGCATATTTCTGAAGTCTCAGAGAAGCAGTGAAGACCACTCATGGGTCTGGGAGGTCCTTTGAGAGCTGGTTC
P O D I P R G H W L O T L K H I S E L L R E A V E D O T H G S C G G P F E S W F 395

1441 CTGTCATTCACTTCGGAGGATGGGCTGAGATGGTGGCAGAGCAATTAAGTATGCTGGCAGCCGAACCCCGAGCCGCTGCTGGGCTTCTGACCTACCGCCCGGCTGATGGG
L F I H G G W A E M V A E O L L M S A E P T A L L W L L A F Y Y G P R D G 435

1561 AGGCAGAGAGCAGAGACTATGGTCAGGTGAAGCGGCTGCGGCACTCTCGGCAATGTCCGAAGCAGCAGCCTCTCAGCCGAGGACCTGCAGAGGTCAGCAGGACGAGGACAGAGC
R O R A O T M V O V K A V L G H L L A M S R S S S L S A O D L O T V A G O G T D 475

1681 ACAGACCTCAGAGCTCTGCACACACCTGATCAGGCACCTTCTCTCAACTTCTGCTCTGGGCTCTGGAGGCGACAGGATCGCTGGGATGTCATCACCTGATGGCTCAGACTGCT
T D L R A P A Q O L I R H L L N F L L W A P G G H T I A W D V I T L M A H T A 515

1801 GAGATAACTCAGAGATCATTTGGCTTTCTTGCAGGACTTTGTACAGTGAATCGTCTTGGCATTGAAGCCCTAGATCAGAAAACCTTGCCCGAGAGCTCTTAAAGAGCTGCGAACT
E I T H E I I G F L D O T L Y R G N R L G I E S P R S E K L A G E L L K E L R T 555

1921 CAAGTCTAGAAGGCAGCAGCGGCTGTGGTGGCGGCTGAGGATCAGGCTCGCCAGGGCCACAGGACAGGTGATGACCTGTGGCCAGCATTTGTGGAGTAAGTGCCTCGCTGGCG
Q V -

2041 TGTGAATGAGCTGTACACATCTTGGGACAATCTGCTAGTATCTATTTTACAAAATGCAGAGCCAGGTCCTCAGCCGAGACTCAGTCAGACATGTTCACTAATGACTCAAGTGAGCTT

2161 CGGTACTCTGGTGGCGCGCGGCGAGCCGTCAGCTTGATAATTAAGCAAGAGGCTGGGTGGGAGAACAGGTTCTAGTCTTTTACCAAGTCAAGCTGCACATCTATTATTTAA

2281 AATTCAAAGTCTTAGAACCAAGAATTGGTCATGAACATTAAAGAAATTAGAGAGAACTAGCTCTTTTAGACTCTTTTAGGAGTCAGGGATCTGGGATAAAGCCACACTGTCTTGC

2401 TGTATGGAGAAATCTTCAAGGGGAGTCAGGTCCTCAGGCTTCCCTGTGCTCCCTGGACTGCTTGCAGGCGCACAGGAGCAGACAGCCAGCCCGGGCTCCGGCACACT

2521 CTTTCCACTCTGTATTGTCTAAATGATGCTAACTGCTACCAAAAGGCCCTTGGACATCAGAGAGCGCGGACGCAAGGTAGAGGATGTGTTCCAGAAACATTAGAAGGCAGGATTAATT

2641 CAGTTAGTTAGTCTCTGTTAAATGGAATGGGAATTGGAATTCCTGATAAGAAATGGCTGGCTGGGTGTCAGTGGCTCACACCTGTGATCCAGCAGCTTTGGAGGCCAAGGCAGGG

2761 GGATTACTTTCAGCCAGGAGTTCCAGACTGCTGGCTAACATGGCAATACCTATCTCTACTAAAAATACAAAATATTATGGGGTGCATGGCATGCATCTGTAACCCAGCTATTCAAGA

2881 GGCTGAGGCATGAGGATCTCTTGAACCGGGAGGTGGGAGTTGTAGTGAGCCGAGATCATGACATGCATCCAGCTGGGCAACAGAGCGAGACCATCTCTAAAAAAGGCATGTTTA

3001 GTGTAACCTCAAGGTTAACAATTTATTCATGTCAGTACAGGGTGTCTTTTCTTTCAGGACATCTCGGAATTTGATTGGTTGATATCTTTTGTGCTATTCTGTTTGTCAAGTGAGTC

3121 AAGACTTGCCTTTTGTCAATTTGATTGTGTGTTTATGCTGAGTCTTGGCTCCGTTTGGGATGAGCAAGTTTGTCTGGATAGAGTTAACTTTAGGGAATTCCTTATTTGGTA

3241 TGTGGCAATGCTAATAGATCCACTGAAGATCTGGAAAAATCCAGGAACTTTTTCACTGAGCCTTCTCTTGAAGATGCTGCAGTCAGAAGGGTGTGCTGGTAAGATTTTGTGGCA

3361 GCTGCCATCATGGTCATTGCCTTCATATAACATGCTTCTGCTCATGGTCATTGCTTTCATATAACATGCTTCTGCTCCATCATGATCCTTGCTTCATATAACAAACATGCTTCTGTCAGA

3481 GGTGTTGGGTGTGAAGAGGAGCTGCATGCTTCACTGGAGTTGAGGGCTCTCTGTCTGACTTAAAGCAAGAACTTGTGGCTGGGCAATGGAAGCTGTGACTCTCTGTGGACATGGTG

3601 GCAGCAGGGAACCCCTAGAGCAGGGGCGCATGGGACAGGCTCTGTTGTGGAGGACTCTGGGACAGTCTCCACCTGTCTGTGCTGTGTACAGGGTTGGCCTTCTCTCC

3721 TCCCTGCGCAGGCTCTGCCATGCCCTTCTTCTCTCTCTGGGACTGGTGAAGCTAGGCATCGGAAGACTTCTTCACTGCTGGAAGCCCTGACCTCGGCCATCTGCAGAACTCTC

3841 CCAGTCTCTTACAGCTCGCGAGTCTCTCAGCGGTGGGTGGAGCGGCTTGGCGTGGTCTTCTGGGACGCGAGGTTCTGGTGGGAGGACTGTCCCTCTGGGAGCTGGCAC

3961 TGAAGTGCCTGCTGGCTTCACTGGCCCTTGGCCCTTCCCAAGCTGAGAGATGCTCAAGAGTGGGAGCTGGGGAGCCACCCCTCGGCCATCTCTCCACCTCCAAGACAGGTGGCG

4081 CCGGGCAGGCACTCTTAAAGCCACCTCCCTCTTGTGTGCTTCTGATTTGGCAAGCTGGGAGCTGCCACGGGAAGGAATGGCATGAGATGCTGGCGGGGACCGCGCTGGCA

4201 GGGGGCTTGACGGCTTGGCGGGCTGGGACAGGGGACCGCGAGGAGGACGAGTGGCAAGGCTGAAGCCACCTTGAAGGAACCTGGACCAAGCTCTTCAAGAGTGGCAGAGGCTC

4321 TGGAACTGACCTTACTCTAGCAGGAGTTTTGTAGACTCTCCCTGATAGTTAGTTTTGATAAAGCATGCTGGTAAACCACTACCTCAGAGAGAGCCAAAAATACAGAGAGGCGG

4441 AGAGCGCCCTCCAAACAGGCTGTTATCCCTGGACTCCGTGACATCTGTGGAATTTTTAGCTCTTAAAAATCTGTAATTTGTGTCTATTTTTCATCTCTAATAAAGCTTCAGTT

4561 GCACCT

100% Poly (A) tail

TABLE 1 Distribution of the frequency of plasmids after selection of pools

Pool	Selective agent	Plasmid identification number								Others	Total
		1	2	3	4	5	8	12	14		
1	Mitomycin C	10	6	2	1	1	—	2	—	14	36
	Diepoxybutane	8	—	11	—	—	—	8	—	9	36
2	Mitomycin C	—	3	16	—	—	—	—	2	15	36
	Diepoxybutane	—	—	25	—	—	—	—	3	8	36
3	Mitomycin C	—	2	2	7	3	11	—	—	11	36
	Diepoxybutane	—	—	—	5	2	26	—	—	3	36
Complementation		—	—	+	+	—	+	—	—		216

The number of times each plasmid was recovered from each pool of selected cells is indicated; a blank means that the plasmid was not recovered in that pool. For complementation, plus and minus signs refer to complementation of the mitomycin C and diepoxybutane hypersensitivity of HSC536N cells as described in Table 2. The transfer of DNA into human cells is inefficient²⁶, and a large number of cells is required to obtain a representative transfection of a complex library. To solve this problem, EBV-transformed lymphocytes (lymphoblasts) were used in the cloning strategy, as these cells grow in suspension and large numbers of cells can readily be transfected¹⁴. The HSC536N cell line has an integrated pSV2neo marker²⁷ introduced through transfection with subsequent selection. Such cells seem to have a greater efficiency in the uptake of DNA, and therefore serve as more efficient recipients in subsequent transfections (unpublished observations). HSC536N cells are ~20- to 30-fold more sensitive to mitomycin C and diepoxybutane than normal cell lines, and ~2- to 3-fold more than other FA cell lines³⁵. Three independent pools of HSC536N cells were transfected with the cDNA expression library and selected through continuous exposure first to mitomycin C and, after outgrowth of survivors, then to diepoxybutane. This dual selection strategy takes advantage of the fact that both agents are metabolized through different pathways during cellular intoxication^{28,29} and helps selection to be highly stringent. The pREP4 cDNA library was transfected into HSC536N cells using Lipofectin (BRL). Briefly, 2×10^7 lymphoblast cells in logarithmic growth phase were pelleted, washed twice in serum-free medium, and resuspended in 3 ml of serum-free medium containing 30 μ g plasmid DNA and 100 μ g Lipofectin. After incubation for 5 to 7 h, the reaction was stopped by adding 7 ml of complete medium. The next morning the culture was diluted to 30 ml; selection in 200 μ g ml⁻¹ hygromycin B (Sigma) was started 48 h later. Dead cells were removed over a Ficoll cushion (Nycomed) after 7 days and survivors were grown under continuous selection. Pools of cells were then selected continuously in 100 nM mitomycin C until outgrowth of survivors was apparent (about 4 weeks). The cells were washed free of mitomycin C and further selected in 1 μ M diepoxybutane until outgrowth (about 2 weeks). Following each selection, plasmid DNA was extracted from the cells through alkaline lysis and transfected into *E. coli* DH10B (ref. 15). Plasmids from individual colonies were mapped with restriction enzymes and grouped as described in the text.

that led to a truncated peptide of 44 residues (data not shown). No other mutations were detected in the coding region of the *FACC* cDNA of these two cell lines; the other mutation is probably in the 5' UTR, 3' UTR or promoter regions of the gene. The conclusion that these two cell lines belong to group C can be tested through complementation analysis using the cloned *FACC* cDNA. This assay is a simple alternative to earlier techniques based on somatic cell hybridization¹⁰ for establishing the complementation group status of unknown FA cell lines; it may be useful for identifying FA(C) cell lines in a wider search for *FACC* mutations.

Discussion

Although the cloning of human genes through functional complementation of cellular defects aims directly at the genes

responsible for the aberrant function, methods using genomic DNA are labour-intensive, involving the generation of individually tailored libraries, and have consequently been used in only a few instances²². The strategy of using an EBV-based expression library obviates most of these considerations and facilitates the rapid and efficient identification of complementing cDNAs. A key factor to the success of this approach is the enhancement of transfection efficiencies with EBV shuttle vectors^{13,15}, because stable expression is independent of the integration event necessary for conventional expression vectors. EBV-based expression libraries can in principle be used to clone cDNAs defective in any human cell line for which a selection

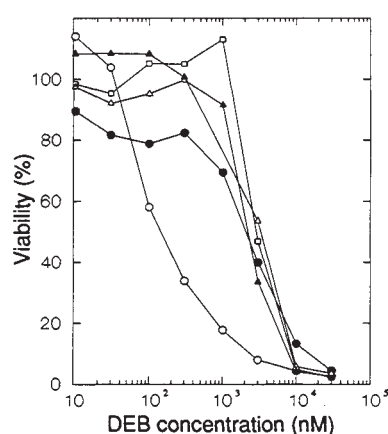


FIG. 2 Analysis of cellular diepoxybutane (DEB) sensitivity. Plot of cellular viability with respect to untreated cells following growth in DEB for the normal control cell line HSC93 transfected with pREP4 vector plasmid alone (●), and HSC536N transfected with either pREP4 (○), pFAC3 (▲), pFAC4 (△), or pFAC8 (□). Candidate cDNAs were transfected into HSC536N cells as described in Table 1. Cellular sensitivity to DEB was assayed as described in Table 2.

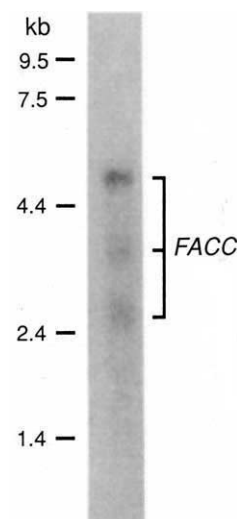


FIG. 3 Northern blot analysis of *FACC* expression. Three *FACC* transcripts are detected, similar in size to the cloned cDNAs at 2.3, 3.2 and 4.6 kb. METHODS. A 5 μ g aliquot of the poly(A)⁺ RNA purified for the cDNA library construction (Fig. 1) was electrophoresed through a 1.2% agarose-formaldehyde gel and transferred to a Hybond N⁺ membrane (Amersham) according to manufacturer's recommendations. The *Bam*HI fragment of pFAC4 was labelled with [α -³²P]dCTP through random priming for use as a probe³².

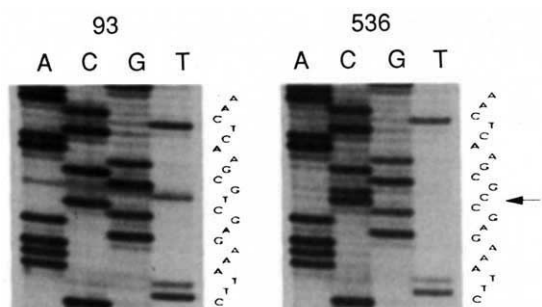


FIG. 4 A T-to-C transition at base 1,913 in HSC536N cells. The respective cell lines and sequencing reactions are shown along the top of the autoradiograph. The *FACC* cDNA sequence and the location of the mutation are indicated down the side of each figure. PCR products from a number of independent amplifications were pooled and sequenced directly to eliminate *Taq* polymerase errors as a source of sequence variation. METHODS. For sequence analysis, asymmetric PCR of reverse-transcribed RNA, (ref. 34) with the FAC-A1 (CGCTGAGTGTGCCGACCATTTCTTC, corresponding to base 184 (5' end of the cDNA)) and FAC-A4 (CCTGTTCTCCACCC-AGGCCTTTTC, corresponding to base 2,236 (3' end of the cDNA)) primer pairs was used to amplify the *FACC* coding region from poly(A)⁺ RNA derived from the indicated cell lines. The thermal profile used was 96° for 20s, then 72° for 120 s, for 40 cycles. PCR products from 4 independent amplifications were pooled, residual primers removed and then sequenced directly using nested internal primers spaced at 250-bp intervals.

scheme can be devised, including those from other DNA repair disorders, those for which metabolic selection is available or those in which selection can be achieved through cell sorting or panning techniques.

FACC transcripts were detected in a wide variety of tissues and cell lines using PCR with reverse-transcribed RNA, indicating that the gene is ubiquitously expressed (data not shown). As these include tissues that can regenerate, such as liver, spleen and fibroblast cultures, as well as those that are not replicating, such as nerve, brain and muscle, a direct role for *FACC* in growth control is unlikely. Characterization of the precise role of *FACC* will be facilitated by the construction of isogenic cell lines, with and without *FACC*. This could lead to therapeutic strategies for patients using pharmacological agents devised to correct the defective function of *FACC*. The identification of *FACC* will also lead to improved genetic diagnosis of Fanconi's

TABLE 2 Assays for cellular diepoxybutane and mitomycin C sensitivity

	Cell line/ plasmid	FA group	EC ₅₀	
			Diepoxybutane (nM)	Mitomycin (nM)
a	HSC93/pREP4		2,900 ± 700	160 ± 30
	HSC536N/pREP4	C	150 ± 20	19 ± 3
	/pFAC3	C	3,000 ± 400	260 ± 40
	/pFAC4	C	2,500 ± 400	130 ± 20
	/pFAB8	C	3,000 ± 600	180 ± 30
b	HSC93/pREP4		1,600 ± 300	150 ± 30
	HSC720T/pREP4	A	46 ± 5	6 ± 1
	/pFAC3	A	61 ± 6	13 ± 6
	HSC230N/pREP4	B	130 ± 20	13 ± 1
	/pFAC3	B	50 ± 9	26 ± 4
	HSC536N/pREP4	C	11 ± 2	11 ± 1
	/pFAC3	C	2,100 ± 300	240 ± 30
	HSC62N/pREP4	D	110 ± 10	8 ± 1
	/pFAC3	D	120 ± 50	19 ± 3

The EC₅₀ (the effective drug concentration giving a 50% reduction in cell viability) and associated standard deviation for each cell line are indicated. HSC93 is a normal control cell line. Data in *a* and *b* are derived from independent experiments. In *b*, pFAC3, pFAC4 and pFAC8 were separately introduced into each cell line. The results were similar for all three plasmids but only the data for pFAC3 is presented. Cellular sensitivity to diepoxybutane and mitomycin C was assayed by plating cells in logarithmic growth at a density of 1.5×10^5 per ml in 96-well microtitre plates. Increasing concentrations of either mitomycin C or diepoxybutane were added in replicates of 8 wells and, after incubation for 5 to 7 days, cellular viability was assayed using 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl-ester (Molecular Probes) as a probe specific for intracellular pH (ref. 30). The data were fitted to a dose-response curve from which the EC₅₀ was calculated.

anaemia and to a better understanding of the relationship between symptoms in patients and their genotype. The severe progressive bone marrow failure in these patients is at present treated by bone marrow transplantation^{7,23}, and although success rates are comparable with those of other patients, bone marrow transplantation in Fanconi's anaemia remains a formidable challenge²⁴. Cloning the Fanconi's anaemia genes is a step towards treatment through gene replacement therapy, which is now being tested in adenosine deaminase deficiency²⁵. □

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A novel heterodimeric cysteine protease is required for interleukin-1 β processing in monocytes

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Interleukin-1 β (IL-1 β)-converting enzyme cleaves the IL-1 β precursor to mature IL-1 β , an important mediator of inflammation. The identification of the enzyme as a unique cysteine protease and the design of potent peptide aldehyde inhibitors are described. Purification and cloning of the complementary DNA indicates that IL-1 β -converting enzyme is composed of two nonidentical subunits that are derived from a single proenzyme, possibly by autoproteolysis. Selective inhibition of the enzyme in human blood monocytes blocks production of mature IL-1 β , indicating that it is a potential therapeutic target.

INTERLEUKIN-1 (IL-1) is a major mediator in the pathogenesis of chronic and acute inflammatory disease. Evidence that IL-1 is an excellent target for therapeutic intervention has been obtained using macromolecules acting as receptor antagonists (IL-1RA (refs 1–4), soluble IL-1R (ref. 5), anti-IL-1R monoclonal antibodies⁶). But, attempts to identify low-molecular-weight receptor antagonists suitable for oral therapy have been uniformly unsuccessful. Thus another approach, amenable to the design and synthesis of small molecules, is needed.

IL-1 β , the predominant form of IL-1 released by human monocytes, is synthesized as an inactive 33K or 31K (M_r 33,000 or 31,000) precursor (pIL-1 β)^{7,8}. The fully active 17.5K mature form of IL-1 β (mIL-1 β) begins at Ala 117 and therefore seems to result from processing between Asp 116 and Ala 117 (refs 9, 10). This unique site for prohormone processing is located in an evolutionarily conserved sequence in all known IL-1 β molecules¹¹. An IL-1 β -converting enzyme (ICE) activity has been identified in monocytes and THP.1 cells which cleaves pIL-1 β at Asp 116–Ala 117 as well as Asp 27–Gly 28 to yield products of 17.5K and 28K, respectively^{12,13}. Cleavage at each site is dependent on aspartic acid in the P₁ position^{12,14,15}. Moreover, ICE seems to be a pIL-1 β -specific processing enzyme because it does not cleave IL-1 α or several other proteins containing many Asp–X bonds¹⁴. This unique specificity should allow the development of selective inhibitors of ICE.

Here we describe the purification, cloning, specificity, and investigations into the catalytic mechanism of ICE. Potent and selective inhibitors have also been used to affinity-purify active

enzyme to homogeneity from THP.1 cell lysates, and to prove the role of ICE in the processing of pIL-1 β .

Purification

ICE was purified from the cytosol of the human monocytic cell line THP.1 by a three-step high-performance liquid chromatography (HPLC) procedure. Sequential fractionation of the cytosolic material over DEAE-5PW and SP-5PW yielded congruent single peaks of ICE activity as measured by cleavage of radiolabelled human pIL-1 β ¹², a 14-amino-acid peptide spanning the pIL-1 β cleavage site¹⁴, or the fluorogenic substrate described below. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the SP-5PW peak fractions indicated that two polypeptides with M_r of 22K and 10K consistently chromatographed with ICE activity (Fig. 1a). Several preparations purified on SP-5PW also contained small amounts of a protein that migrated with an M_r of 24K (p24) on SDS-PAGE (results not shown). Each fraction containing ICE activity was individually fractionated by reversed-phase HPLC. Two peaks, neither of which retained enzymatic activity (not shown), were observed in direct proportion to the amount of ICE activity injected onto the column. Analysis of the peaks by SDS-PAGE showed that the first peak contained the 22K protein, and the second the 10K protein, in homogeneous form (Fig. 1b). Roughly 700 pmol of each protein were purified from 1×10^{11} cells.

Relative molecular masses for the 22K and 10K proteins were determined by on-line liquid chromatography/mass spectral analysis using electrospray ionization¹⁵. The average M_r values of the purified proteins were 19,866 (Fig. 1c) and 10,248 (Fig. 1d), respectively, and these were designated p20 and p10.

Substrate specificity

Peptides that span the cleavage site of pIL-1 β are suitable substrates for ICE and have been used to define its substrate specificity^{14,16}. Two features of peptide substrates are essential for catalytic recognition by the enzyme. First, there is a strong preference for aspartic acid adjacent to the cleavage site, in that any substitution of this residue in pIL-1 β and peptide substrates leads to a substantial reduction in the rate of catalysis (>100 -fold)^{14,16}. The results in Fig. 2 indicate that there is an equally stringent requirement (>100 -fold) for four amino-acid residues to the left of the cleavage site, whereas methylamine is sufficient to the right. The results also indicate that broad substitution is tolerated in the P₂ position (peptides 18–24); this has been exploited in the design of peptide substrates and inhibitors described below. The minimal substrate for the enzyme, Ac-Tyr-Val-Ala-Asp-NH-CH₃ (peptide 16; AC, acetyl), is the best peptide substrate yet identified for the enzyme, having a relative

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$V_{\max}/K_m$ comparable to that for pIL-1 β (12 ± 2 versus 10 ± 4 for pIL-1 β).

These features are embodied in the fluorogenic substrate, Ac-Tyr-Val-Ala-Asp-7-AMC (peptide 17; AMC, amino-4-methylcoumarin). Cleavage of this substrate shows Michaelis-Menten kinetics with $K_m = 14 \pm 3 \mu\text{M}$. The continuous, fluorometric assay developed with this substrate has facilitated a detailed investigation of the catalytic mechanism of the enzyme.

Classification as a cysteine protease

ICE is inactivated by thiol alkylating reagents¹³. The enzyme is also inhibited by 1,10-phenanthroline (our unpublished observations); however, the mechanism of inhibition is not due to chelation of an active-site metal, but rather to contamination of the phenanthroline with Cu(II) and concomitant metal-catalysed oxidation of an essential thiol¹⁷. Although such data indicate that the enzyme contains accessible structural and/or catalytic thiol(s), this does not permit assignment of ICE to a protease class.

Three lines of evidence now clearly identify ICE as a cysteine protease. First, and most compelling, is that a diazomethylketone designed with the appropriate active site specificity (Ac-Tyr-Val-Ala-Asp-COCHN₂, inhibitor A) is a potent, competitive, irreversible inhibitor of the enzyme. Diazomethylketones are highly selective, covalent inhibitors of cysteine proteases¹⁸. Addition of ICE to reaction mixtures containing $1 \times K_m$ substrate and 250 nM inhibitor A resulted in time-dependent inhibition of the enzyme (Fig. 3a). Irreversible inhibition was demonstrated by the lack of recovery of activity when saturating levels of substrate ($10 \times K_m$) were added to the completely inhibited reaction. The inhibition is strictly competitive, as indicated by the protection against inactivation observed in the presence of saturating substrate ($70 \times K_m$). The second order rate constant for inactivation, $1.64 \pm 0.03 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, is comparable to those of the best diazomethylketone inhibitors of other cysteine proteases^{18,19}. A 10,000-fold smaller value was obtained for the truncated inhibitor Cbz-Asp-COCHN₂ (Cbz represents carbobenzyloxy group), consistent with substrate specificity studies.

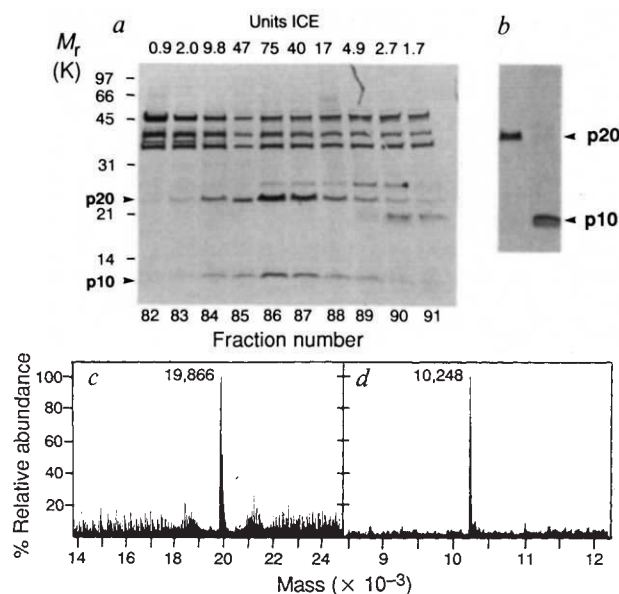


FIG. 1 Identification of ICE proteins. *a*, SDS-PAGE (17–27%) and silver staining of SP-5PW ICE peak fractions (82–91). ICE activity in each fraction as measured in the fluorogenic assay is shown over each lane. Arrows indicate the positions of the 22K and 10K bands that correlated with ICE activity. *b*, SDS-PAGE and silver staining of p20 and p10 ICE proteins purified by reversed-phase HPLC (RP-HPLC). *c*, *d*, The mass of each subunit was determined by mass spectrometry¹⁵; *c*, 22K subunit (p20); and *d*, 10K subunit (p10).

METHODS. *a*, *b*, ICE activity was measured as described previously¹⁴ and in Fig. 2 legend. THP.1 cells were grown in batch fermenters in Iscove's medium supplemented with 9% horse serum and 0.1–0.3% F68 pluronic, washed in PBS, and broken by dounce homogenization in hypotonic buffer containing 25 mM HEPES, pH 7.5, 5 mM MgCl₂, and 1 mM EGTA, 1 mM PMSF and 10 $\mu\text{g ml}^{-1}$ pepstatin and leupeptin. After differential centrifugation, the cytosolic extract was clarified by filtration through a 0.22- μm filter, dialysed overnight (5 °C) with 20 mM Tris, pH 7.8, 10% sucrose, 0.1% CHAPS, and 2 mM DTT (TSCD buffer). Total protein (3–5 g), corresponding to 1,000 ml cytosolic extract, was applied to a 475-ml bed volume DEAE-5PW HPLC column and eluted with TSCD buffer and a gradient of 0.4 M NaCl and 240 mM Tris-HCl (pH 7.8). Fractions from the DEAE column (50–150 mg) were pooled, diluted with an equal volume of HSCD buffer (contains 25 mM HEPES instead of Tris), adjusted to pH 7.0, applied to a 150-ml bed volume SP-5PW HPLC column and eluted with KCl. Aliquots of SP-5PW fractions were injected onto a narrow bore C4 RP-HPLC column and eluted with a linear gradient of 0.06% trifluoroacetic acid in acetonitrile/H₂O. *c*, *d*, RP-HPLC-purified p20 and p10 (5 pmol) were analysed by capillary liquid chromatography coupled to a Finnigan MAT TSQ-700 triple sector quadrupole mass spectrometer equipped with an electrospray ion source¹⁵.

Amino-terminal truncations

Peptide	P7	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'	P6'	P7'	Relative $V_{\max}/K_m$
1	Asn	Glu	Ala	Tyr	Val	His	Asp	Ala	Pro	Val	Arg	Ser	Leu	Asn	1.00 $\pm$ 0.05
2		Glu	Ala	Tyr	Val	His	Asp	Ala	Pro	Val	Arg	Ser	Leu	Asn	0.64 $\pm$ 0.01
3			Ala	Tyr	Val	His	Asp	Ala	Pro	Val	Arg	Ser	Leu	Asn	0.29 $\pm$ 0.02
4				Tyr	Val	His	Asp	Ala	Pro	Val	Arg	Ser	Leu	Asn	0.12 $\pm$ 0.00
5				Ac-Tyr	Val	His	Asp	Ala	Pro	Val	Arg	Ser	Leu	Asn	0.81 $\pm$ 0.01
6					Ac-Val	His	Asp	Ala	Pro	Val	Arg	Ser	Leu	Asn	<0.005
7						His	Asp	Ala	Pro	Val	Arg	Ser	Leu	Asn	<0.005
8							Asp	Ala	Pro	Val	Arg	Ser	Leu	Asn	<0.005

Carboxy-terminal truncations

Peptide	P7	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'	P6'	P7'	Relative $V_{\max}/K_m$
1	Asn	Glu	Ala	Tyr	Val	His	Asp	Ala	Pro	Val	Arg	Ser	Leu	Asn	1.00 $\pm$ 0.05
9	Asn	Glu	Ala	Tyr	Val	His	Asp	Ala	Pro	Val	Arg	Ser	Leu		1.22 $\pm$ 0.16
10	Asn	Glu	Ala	Tyr	Val	His	Asp	Ala	Pro	Val	Arg	Ser			0.80 $\pm$ 0.16
11	Asn	Glu	Ala	Tyr	Val	His	Asp	Ala	Pro	Val	Arg				0.88 $\pm$ 0.04
12	Asn	Glu	Ala	Tyr	Val	His	Asp	Ala	Pro	Val					1.02 $\pm$ 0.09
13	Asn	Glu	Ala	Tyr	Val	His	Asp	Ala	Pro						1.25 $\pm$ 0.15
14	Asn	Glu	Ala	Tyr	Val	His	Asp	Ala							<0.05
15	Asn	Glu	Ala	Tyr	Val	His	Asp	Ala-CO-NH ₂							0.93 $\pm$ 0.03
16				Ac-Tyr	Val	Ala	Asp-NH-CH ₃								12.00 $\pm$ 2.00
17				Ac-Tyr	Val	Ala	Asp-AMC								1.9 $\pm$ 0.1

P2 substitutions

Peptide	P7	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'	P6'	P7'	Relative $V_{\max}/K_m$
18				Ac-Tyr	Val	Ala	Asp	Gly	Trp-NH ₂						6.6 $\pm$ 0.1
19				Ac-Tyr	Val	His	Asp	Gly	Trp-NH ₂						5.4 $\pm$ 1
20				Ac-Tyr	Val	Gln	Asp	Gly	Trp-NH ₂						4.0 $\pm$ 0.3
21				Ac-Tyr	Val	Lys	Asp	Gly	Trp-NH ₂						2.5 $\pm$ 0.3
22				Ac-Tyr	Val	Pro	Asp	Gly	Trp-NH ₂						1.6 $\pm$ 0.1
23				Ac-Tyr	Val	Gln	Asp	Gly	Trp-NH ₂						0.59 $\pm$ 0.08
24				Ac-Tyr	Val	Asp	Asp	Gly	Trp-NH ₂						0.34 $\pm$ 0.03

FIG. 2 Substrate specificity studies. Peptides that span the cleavage site of pIL-1 β were evaluated as substrates and used to define the enzyme's minimum recognition sequence. Values for $V_{\max}/K_m$ are expressed relative to peptide 1 and represent the mean $\pm$ s.d. ($n = 2-4$). The results indicate that peptide 16 is the minimum recognition sequence for ICE, and that broad substitution is tolerated in P₂.

METHODS. Peptides 1–16 and 18–24 were synthesized using the Merrifield solid-phase technique, purified by RP-HPLC, and confirmed by mass spectral analysis. The fluorogenic substrate, Ac-Tyr-Val-Ala-Asp-AMC (peptide 17), was prepared by conventional solution-phase peptide synthesis. The enzyme used in these studies was purified 100-fold using DEAE ion-exchange chromatography as described in Fig. 1. Peptide cleavage reactions contained 50 units enzyme (1 unit = 1 pmol AMC per min at saturating concentrations of peptide 17) and were done as previously described¹⁴. In the case of pIL-1 β , substrate (33K) and product (17.5K) were separated by SDS-PAGE¹², and quantitated using a radioanalytical imaging system (Ambis Systems).

Second, inactivation of the enzyme by iodoacetate is competitive with substrate (not shown). Competitive inactivation was also demonstrated with other reagents, including cystamine (2-aminoethanethiol disulphide) and glutathione disulphide. Finally, consistent with the enhanced reactivity of catalytic cysteines found in other cysteine proteases¹⁸, the catalytically essential cysteine reacts with [¹⁴C]iodoacetate more than 10 times faster than do other cysteines or dithiothreitol (not shown).

The selective and competitive inactivation by [¹⁴C]iodoacetate was used to identify the active-site cysteine. On the basis of the rate constant for inactivation of free enzyme by iodoac-

tate ($20 \text{ M}^{-1} \text{ s}^{-1}$), conditions were chosen for 99% inactivation in the absence of substrate and only 2% in the presence of saturating levels ($200 \times K_m$). The labelling patterns shown in Fig. 3b were obtained with enzyme purified 10,000-fold by DEAE-cellulose and sulphydrylpropyl chromatography. Coomassie blue staining of the gel indicated about 10 major proteins, of which only two, p20 and a band of higher M_r , underwent significant ¹⁴C-carboxymethylation. Of these, only p20 was protected by saturating substrate. This established that the catalytically essential cysteine is located in the p20 subunit. Sequencing of a tryptic digest indicated only a single labelled peptide with the sequence Val-Ile-Ile-Ile-Gln-Ala-Cys, with the peak of label coinciding with the cysteine.

Reversible inhibition by a peptide aldehyde

The catalytic mechanism of the enzyme suggested that peptide aldehydes would be attractive transition-state analogue inhibitors²⁰. This was confirmed with the design and synthesis of Ac-Tyr-Val-Ala-Asp-CHO (inhibitor B), which is a potent, competitive, reversible inhibitor of ICE. It is slow binding, as shown by the slow approach to equilibrium when enzyme was added to reaction mixtures containing inhibitor (50 nM) and $1 \times K_m$ substrate (Fig. 4a). Competitive and reversible inhibition was indicated by the recovery of activity observed when saturating levels of substrate ($10 \times K_m$) were added to fully inhibited enzyme. The behaviour of this inhibitor is consistent with a kinetic model in which the rates of association (k_{on}) and dissociation (k_{off}) of enzyme-inhibitor complex are slow compared to rate constants for interaction with the substrate²¹. The k_{on} ($3.8 \pm 0.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) and k_{off} ($2.9 \pm 0.4 \times 10^{-4} \text{ s}^{-1}$) define the overall dissociation constant for the equilibrium: $K_i = k_{off}/k_{on} = 0.76 \pm 0.16 \text{ nM}$. A P_3 -substituted aldehyde used as a control in whole-blood studies (see below), Ac-Tyr-D-Ala-Ala-Asp-CHO (inhibitor C), has a 1,000-fold reduced affinity for the enzyme ($K_i = 1.5 \pm 0.1 \mu\text{M}$).

Affinity purification

Affinity chromatography was used to purify active enzyme to homogeneity and to demonstrate the selectivity of the peptide aldehyde inhibitors. The inhibitor Ac-Tyr-Val-Lys-Asp-CHO (inhibitor D), containing lysine at P_2 , was synthesized as an affinity ligand and shown to have somewhat lower affinity ($K_i = 3 \text{ nM}$) than the original aldehyde, as predicted from substrate studies (Fig. 2, peptide 21).

As anticipated from the enzyme's unusual specificity, the immobilized peptide aldehyde proved to be very selective. Affinity chromatography of a crude THP.1 lysate isolated only two proteins, with M_r values corresponding to p20 and p10 (Fig. 4b). Analysis of affinity-purified enzyme by reversed-phase HPLC and amino-terminal sequence analysis indicated that the subunits are present in equimolar ratio (assuming equal extinction coefficients at 214 nm), and confirmed their identity with those described in Fig. 1. Cleavage of internally labelled pIL-1 β ¹² by affinity-purified ICE yielded mL-1 β with Ala 117 at the N terminus. The affinity chromatography represents a purification of >100,000-fold in a single step. Besides demonstrating the singular specificity of the peptide aldehyde inhibitors, this affinity method provided the first preparations of pure, catalytically active enzyme.

Kinetic evidence that ICE is an oligomer

ICE displays kinetic properties of a dissociable multimeric protein in that the enzyme inactivates on dilution. Dilution of the enzyme by 1,000-fold into a sample containing $1 \times K_m$ substrate results in time-dependent loss of activity ($t_{1/2} = 2.7 \pm 0.4 \text{ h}$; Fig. 4c). Reconcentration (1,000-fold) of the dilute, inactive preparation results in complete recovery of activity (Fig. 4c, inset). The most reasonable explanation for this concentration-dependent behaviour is that catalytically active enzyme is composed of at least two subunits and that both subunits are required for

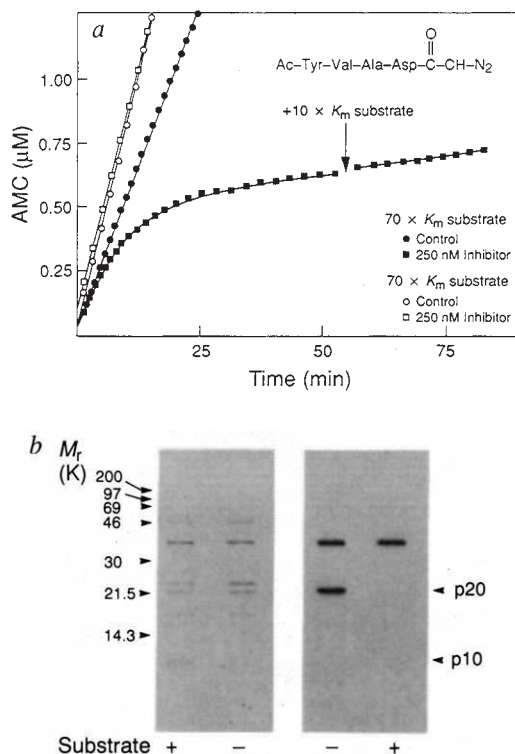


FIG. 3 Evidence that ICE is a cysteine protease. *a*, Inhibition of ICE by the tetrapeptide diazomethylketone, inhibitor A. Addition of peptide A (250 nM) to a reaction mixture containing $1 \times K_m$ substrate resulted in first-order loss of enzyme activity. The solid line is a theoretical fit to the integrated first-order rate equation developed by Morrison²¹, yielding a value for the first-order rate constant of $2 \times 10^{-3} \text{ s}^{-1}$, corresponding to a second-order rate constant of $1.6 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$. The residual activity observed (<3%) represents background cleavage of the substrate due to contaminating proteases. Addition of saturating levels of substrate ($10 \times K_m$) has no influence on the inhibited velocity, indicating irreversible inhibition. To demonstrate competitive inactivation, an identical experiment was done in the presence of saturating substrate ($70 \times K_m$). *b*, Labelling patterns with [¹⁴C]iodoacetate. ICE was labelled with [¹⁴C]iodoacetate in the absence (-) and presence (+) of saturating substrate, and analysed by SDS-PAGE. The left-hand lanes are stained with Coomassie blue; the right-hand lanes are autoradiographed. The missing ¹⁴C-labelled band in the substrate-protected case identifies the p20 subunit as containing the active-site cysteine.

METHODS. *a*, Ac-Tyr-Val-Ala-Asp-COCHN₂, inhibitor A, was prepared as follows: aspartic acid diazomethylketone β -methyl ester was coupled to Asp-Tyr-Val-Ala using dicyclohexylcarbodiimide (DCC) and hydroxybenzotriazole (HOBt), and the product hydrolysed with triethylamine and water to yield the final product. Reactions (500 μl) contained Ac-Tyr-Val-Ala-Asp-AMC (14 μM or 1 mM) and enzyme (25 units) under standard assay conditions. Reactions were monitored continuously in a spectrofluorometer at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. *b*, Labelling was done on sulphydrylpropyl-purified enzyme, in solutions containing 1,400 units ml^{-1} enzyme, 100 μM [¹⁴C]iodoacetate (56 Ci mol^{-1}), in the standard assay buffer¹⁴ (including dithiothreitol) at 25 °C plus or minus 2.8 mM substrate ($200 \times K_m$), for seven half-lives (40 min).

catalytic activity. Dissociation was not observed in the presence of saturating levels of substrate or inhibitor (not shown).

cDNA cloning

N-terminal and internal tryptic peptide sequences from purified p20 or p10 were used to design degenerate oligonucleotide primers for polymerase chain reaction (PCR) amplification of ICE-specific DNA fragments from THP.1 cell cDNA²². Separate PCR products for p20 and p10 were subcloned and used as probes to isolate full-length cDNAs from THP.1 and human monocyte libraries. All of the primary phage isolates hybridized with both probes, demonstrating that both ICE proteins were encoded by the same messenger RNA.

Several cDNAs were sequenced to determine the primary structure of ICE (Fig. 5a, b). There were no significant differences in the cDNA sequences of THP.1 and human monocyte ICE. The longest cDNA insert, which is 1,490 base pairs (bp) long, corresponded to a constitutively expressed, low-abundance transcript (<1:50,000) of 1.6 kilobases (kb) based on northern blot analyses of THP.1 RNA (not shown). The ICE cDNA also hybridized to poly(A) mRNAs of 2.3 and 0.5 kb. All three ICE mRNAs were detected in several non-monocytic cell types including neutrophils and T-lymphocytes, and the Raji B lymphoblastoid cell line (not shown). Five additional nucleotides compared to that of the longest insert were detected by cDNA primer extension (not shown), suggesting that ICE mRNA has a very short 5' untranslated region (UTR) or secondary structure which limits 5' extension. The most favourable Kozak consensus sequence (GCCATGG)²³ is at nucleotide 8 and is followed by a long open reading frame (ORF) of 1,212 nucleotides, that terminates with the ochre codon, TAA, at nt 1,220. The cDNA encodes a predicted proenzyme of 404 amino acids (M_r , 45,158).

A protein with an M_r of 45K (p45) results from *in vitro* transcription and translation of the full-length ICE cDNA (Fig. 6b, lane 1). Two types of ICE cDNAs were identified which differed in the sequence and length of the 3'UTR, suggesting that ICE transcripts are alternatively spliced (not shown).

Primary structure

Amino-acid sequence and mass spectrometry information were used to delineate the regions in p45 corresponding to p20 and p10. Sequence analysis also indicated that p24 was an alternately processed form of p20 (Fig. 5a, b). From Edman sequencing, the N termini of p24 and p20 are located at Ser 104 and Asn 120, respectively. The N-terminal region of p45 encodes a 119-residue propeptide, containing three cysteines, which lacks the characteristics of a hydrophobic signal sequence. Its function is not known but it is clear from the affinity purification results that it is not required for enzymatic activity (Fig. 4b). Asp 297 was predicted as the C-terminus of p20 based on the M_r of the subunit (Fig. 1c), identification by fast-atom bombardment mass spectrometry of an appropriately sized Asp-N peptide (Asp 288–Asp 297), and sequence analysis of this peptide by MS/MS²⁴. The conclusion that this peptide lies at the terminus of p20 is consistent with the inability of Asp-N to cleave C-terminal aspartic acid residues. The catalytic cysteine identified by active-site labelling, Cys 285, lies within 13 residues of the C terminus of p20.

Sequence analysis of the purified protein and cDNA demonstrated that p10 begins at Ala 317 and terminates with the end of the ORF at His 404. These assignments indicate that a 19-residue spacer separates the C terminus of p20 from the N terminus of p10. Neither this fragment nor the propeptide has been found in purified ICE preparations.

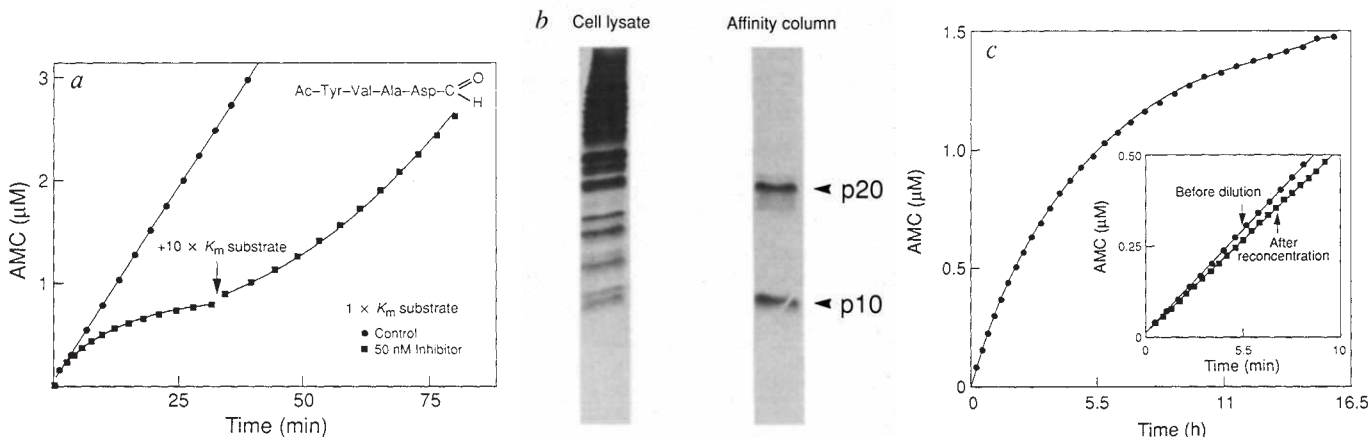


FIG. 4 Potency and selectivity of tetrapeptide aldehydes and evidence that ICE is an oligomer. *a*, Addition of the tetrapeptide aldehyde, peptide B, (50 nM) to a reaction mixture containing $1 \times K_m$ substrate resulted in time-dependent loss of enzyme activity. Addition of saturating levels of substrate ($10 \times K_m$) to the inhibited reaction resulted in slow, first-order recovery of activity, indicating reversible and competitive inhibition. *b*, Affinity chromatography. Silver-stained SDS-PAGE of THP.1 cell lysate applied to the affinity column, and pure ICE eluted by free inhibitor. The procedure gave ~50% recovery of enzymatic activity, and a purification of >100,000-fold. *c*, Reversible inactivation of ICE by dilution. Enzyme (50 μl , 5 units μl^{-1}) diluted 1,000-fold into reaction mixtures containing 14 μM Ac-Tyr-Val-Ala-Asp-AMC ($1 \times K_m$) undergoes reversible dissociation. The solid line is theoretical for a first-order loss of activity with a rate constant of 0.004 min^{-1} (half life, 2.9 h). Less than 2% of the total substrate was consumed over the reaction period. An identical experiment in the absence of sucrose and CHAPS results in a 4-fold faster inactivation rate and both of these components act independently to stabilize the enzyme (not shown). Complete recovery of activity is achieved on 1,000-fold re-concentration of the enzyme. The inset compares the initial velocities before dilution ($V_0 = 0.057 \mu\text{M AMC min}^{-1}$) and after concentration ($V_0 = 0.051 \mu\text{M AMC min}^{-1}$).

METHODS. *a*, Ac-Tyr-Val-Ala-Asp-CHO, peptide B, was prepared as follows:

Alloc-L-aspartic acid β -*t*-butyl ester (Alloc is allyloxycarbonyl) was reduced to the corresponding alcohol through a mixed anhydride using sodium borohydride. The alcohol was oxidized to the aldehyde and converted without purification to the *O*-benzylacetal using trifluoroacetic acid and benzyl alcohol. The Alloc group was removed and the resulting amine coupled *in situ* to Ac-Tyr-Val-Ala using ethyldimethylaminopropylcarbodiimide hydroxybenzotriazole. Hydrogenolysis provided the desired peptide aldehyde. Reactions (500 μl) contained Ac-Tyr-Val-Ala-Asp-AMC (14 μM or 1,000 μM) and enzyme (25 units) under standard assay conditions. The k_{on} , k_{off} and K_i , were calculated according to ref. 21. *b*, The affinity matrix was prepared by coupling the inhibitor D, protected as its dimethyl acetal, to crosslinked Sepharose 4B by established methods³². A lysate from THP.1 cells¹² was equilibrated with an equal volume of affinity gel for 5 h at 4 $^{\circ}\text{C}$, the gel was washed in a column with 100 volumes standard assay buffer, and the enzyme recovered by exchange with 200 μM free inhibitor for 24 h followed by elution with two column volumes of buffer. The enzyme was reactivated by treatment with hydroxylamine and glutathione disulphide, followed by reduction with DTT. *c*, After 20 h, the diluted enzyme (50 ml) was re-concentrated to 50 μl using an Amicon ultrafiltration apparatus (YM10 membrane) and a Centricon-10.

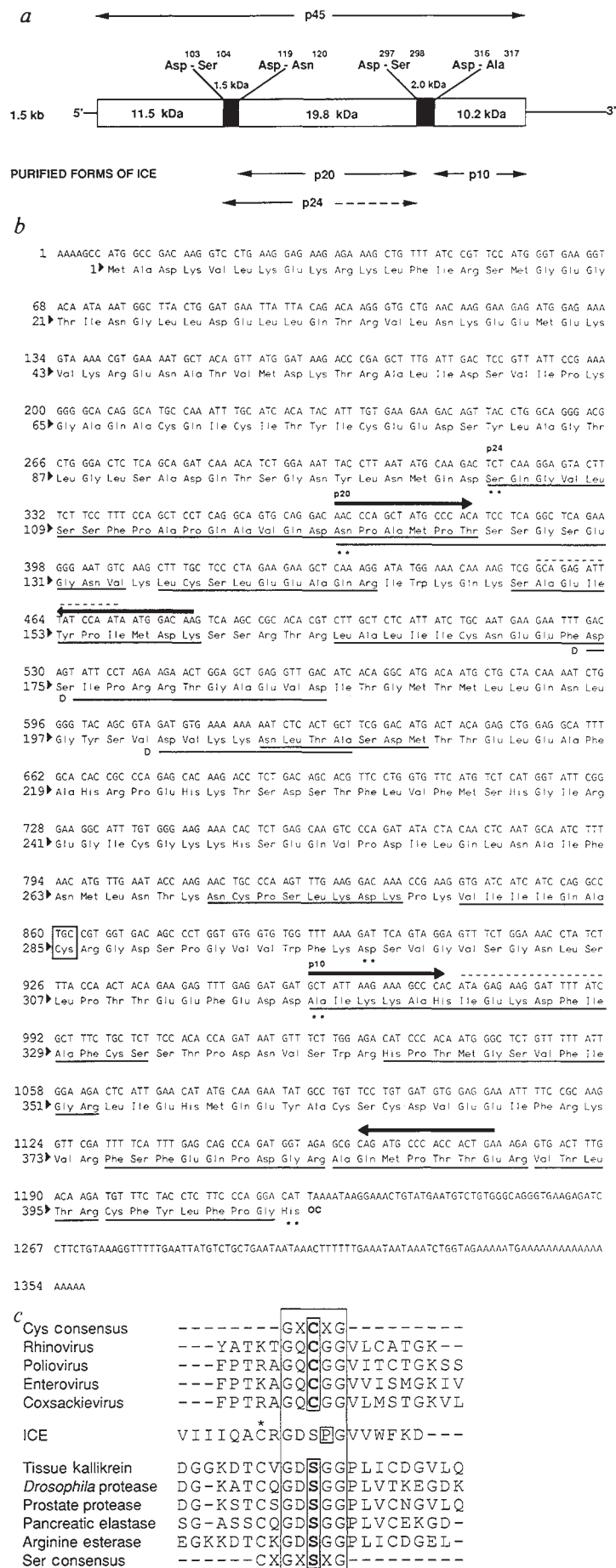


FIG. 5 Organization of the human ICE cDNA. **a**, The predicted ORF encoding the ICE proenzyme (p45) is represented by a rectangle. 5' and 3' UTR sequences are shown as solid bars. The four ICE-like cleavage sites flanking the known forms of ICE (p24, p20 and p10) are indicated. The C terminus of p24 has not been determined and is represented by a dashed arrow. **b**, Nucleotide and deduced amino-acid sequence of ICE. Nucleotides are numbered 5' to 3'. The ochre termination codon is designated OC. Primers used for amplification are denoted by bold arrows. Oligonucleotide probes used to characterize the PCR products are indicated by dashed lines. Deduced sequences confirmed by Edman sequencing are underlined (only a subset are shown for clarity). Asp-N-derived peptides are denoted by D. The N termini of p24, p20 and p10 and the C termini of p20 and p10 are indicated by double asterisks. The active-site Cys is boxed. **c**, Alignments of ICE with cellular serine and viral cysteine proteases. ICE is aligned with the highest scoring sequences from the final Profile searches. Serine and viral cysteine active-site consensus sequences^{25,26} and the corresponding sequence in ICE are boxed. Shaded boxes indicate the active-site cysteine or serine residues. The asterisk denotes the catalytic Cys 285 of ICE. Serine protease sequences are tissue kallikrein from mouse (A05308), a serine protease from *Drosophila melanogaster* (PS0049), a prostate-specific protease (S03604), pancreatic elastase (A23473), and arginine esterase (S00613). Viral cysteine protease sequences are coxsackie virus (GNNYA9), bovine enterovirus (GNNYBE), poliovirus type 1 (GNNY1P) and human rhinovirus type 14 (GNNYH4). **METHODS.** **a** and **b**, p20 and p10 were reduced, alkylated and sequenced as described previously³³. For p20, the N-terminal primer, GAYCCNGCNATGCCNAC, was 128 times degenerate and the internal primer, TTRCCATDATNGGRTA, 48 times degenerate (Stanford code³⁸). For p10, the N-terminal primer, GCNATHAARAARGCNCA, was 192 times and the internal primer, GTYTACGNTGNTGNCT, 128 times degenerate. Single-stranded THP 1 cDNA was synthesized from poly(A)⁺ mRNA and used as a PCR template as described²². For p20, a PCR product of 116 bp was confirmed as ICE-specific by hybridization with the oligonucleotide ATIGGRTAATYTCTGCR. A 221-bp PCR product was verified for p10, with the probe ATIGARAARGAYTTYATIGC. Probes were 5' end-labelled and hybridized at 33 °C (15 to 20 °C below *T_m*) as described previously³⁴. The subcloned PCR products were sequenced by the chain termination method³⁵ and used as hybridization probes in separate screenings of commercial (Clontech) THP.1 (λgt10) or human monocyte (λgt11) cDNA libraries³⁵. Positive inserts were subcloned and sequenced on both strands³⁵. **c**, Homology searches were conducted with the ICE nucleotide and protein sequences against Genbank³⁶ and PIR³⁷ using the GCG package³⁸. A non-redundant database³⁹ was used to generate distinct profiles⁴⁰ representing the catalytic regions of cellular serine and viral cysteine proteases. These profiles, designed using alignments suggested in ref. 26, were used to search for similar regions in the primary sequence of ICE. PIR sequence identifiers are bracketed.

The theoretical mass for p20 (Asn 120–Asp 297), assuming all five cysteines including the active-site Cys 285 are reduced, is 19,844. The theoretical mass for p10 (Ala 317–His 404), assuming that all four cysteines are reduced, is 10,244. The mass values determined for p20 (19,866) and p10 (10,248) by on-line liquid chromatography/mass spectral analysis (Fig. 1c, d) are within 0.11 and 0.04%, respectively, of the theoretical values and are in keeping with the accuracy of this technique for proteins of this size (0.01–0.20%)¹⁵. These results indicate that neither ICE subunit undergoes significant post-translational modification (for example, farnesylation). But the acidic conditions used for reversed-phase HPLC might remove acid-labile groups (such as phosphate). We hypothesize that in non-stimulated THP.1 cells ICE, like pIL-1 β ¹¹, is in the cytosol.

The sequence of ICE is not homologous to any known protein, including cellular cysteine proteases. But Ser 289 of ICE and adjacent residues readily aligns with the consensus sequence (Gly-X-Ser/Cys-X-Gly)^{25,26} corresponding to the catalytic regions of serine and viral cysteine proteases (Fig. 5c). Using this alignment, the catalytic cysteine of ICE, Cys 285, aligns with a conserved cysteine which is normally disulphide-linked in serine proteases.

Functional expression

To verify further the identity of the ICE cDNA, the 1.5-kb insert was cloned into an expression vector, transfected into COS-7 cells, and assayed for ICE activity. Lysates from ICE-transfected cells, but not those from cells transfected with the expression vector alone, cleaved >80% of the pIL-1 β to 17.5 and 28K products (Fig. 6a, lanes 1 and 2). The 17.5K cleavage product comigrated with mIL-1 β produced by incubation of the substrate with affinity-purified ICE (Fig. 6a, lanes 4 and 5) and was completely inhibited by inhibitor B (Fig. 6a, lane 3). The substrate specificity of the expressed ICE activity was verified by incubating lysates with a pIL-1 β cleavage site mutant (Asp 116→Ala) which cannot be cleaved at Ala 116–Ala 117 by ICE¹². As with native ICE, the activity from the transfectants cleaves the mutant substrate to a 28K product but not to mIL-1 β (Fig. 6a, lanes 6 and 7)¹².

Processing of p45

The primary sequence of ICE revealed that the N termini of p24 and p10 as well as the N and C termini of p20 result from cleavage of Asp–X bonds, where X equals Ser 104, Asn 120, Ser 298 or Ala 317 (Fig. 5a). Human ICE requires Asp in P₁ and prefers small hydrophobic residues (Gly>Ala>Val) in P_{1'} (ref. 14). But not all proteins with Asp–X bonds are cleaved

by ICE, human and murine pIL-1 β remaining the only known substrates¹⁴. Therefore, it was of interest to determine whether active ICE could cleave the proenzyme. The primary ICE translation product, p45, is stable in rabbit reticulocyte lysates for at least 24 h (Fig. 6b, lane 1). On the addition of affinity-purified ICE, p45 was cleaved to yield multiple products (Fig. 6b, lane 2), all of which are congruent with the purified forms of ICE (p24, p20, p10; Fig. 5a) or intermediates predicted to result from single cleavages of the proenzyme. At an inhibitor concentration of 200 nM, cleavage of p45 by affinity-purified ICE was blocked by inhibitor B (K_i =0.7 nM), but not inhibitor C (K_i =1 μ M; Fig. 6b, lanes 3 and 4), indicating that processing was not due to a contaminating protease.

Effect of peptide aldehyde on mIL-1 β release

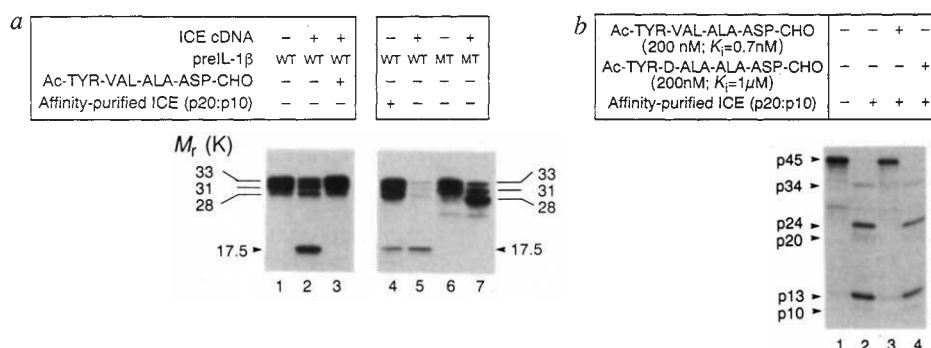
The potency and selectivity of the tetrapeptide aldehyde, inhibitor B, make it a useful inhibitor with which to test the processing function of ICE in intact blood monocytes. Pulse-chase experiments (Fig. 7a) demonstrated that human blood monocytes rapidly synthesize pIL-1 β when stimulated with heat-killed *Staphylococcus aureus*²⁷. Intracellular pIL-1 β chases directly into plasma as mIL-1 β with a half-life of 1.5–2.0 h at 30–95% efficiency, depending on the blood donor.

Titration of inhibitor B into human blood caused a dose-dependent decrease in the plasma level of mIL-1 β with a 50% inhibitory concentration (IC₅₀) of ~4 μ M (Fig. 7b, d). Although the IC₅₀ is 5,000 $\times$ K_i , this is consistent with a cytosolic location for ICE and our finding that the peptide aldehyde penetrates cells poorly (unpublished observation). Inhibition was accompanied by a parallel increase in plasma pIL-1 β , such that the total amount of secreted IL-1 β (mIL-1 β +pIL-1 β) remained constant within experimental error (Fig. 7d). The inhibitor had no effect on the intracellular amounts of immunoprecipitable pIL-1 β (not shown).

Several controls indicate the specificity of the inhibition. First, the control aldehyde, inhibitor C (K_i =1.5 μ M), was considerably less potent (IC₅₀>100 μ M; Fig. 7c, e). Second, other protease inhibitors capable of penetrating cell membranes, including pepstatin, E-64 ((N-(L-3-triscarboxyoxiran-2-carbonyl)-L-leucyl)-amido(4-guanido)butane), a neutrophil elastase inhibitor¹², and phosphoramidon were without effect at concentrations up to 100 μ M. Finally, concentrations of inhibitor B up to 100 μ M had no effect on the plasma levels of IL-6, IL-8 and tumour necrosis factor (TNF- α), the latter two of which are proteolytically processed^{28,29}. These results indicate that ICE alone is responsible for activation of pIL-1 β .

FIG. 6 Functional expression and processing of ICE. **a**, ICE activity in lysates from transiently transfected COS-7 cells. Cells transfected with the ICE cDNA (lanes 2, 3, 5, 7) or control vector (lanes 1, 4, 6) were incubated with wild-type (WT) pIL-1 β (lanes 1–5) or the mutant (MT) Ala 116 pIL-1 β (lanes 6 and 7) synthesized by *in vitro* translation¹². ICE-transfected cell lysates were also incubated with the inhibitor B, before substrate addition (lane 3). As a control, affinity-purified ICE (2 units) was added to a lysate from cells transfected with the vector alone (lane 4). The sizes of the pIL-1 β (33 or 31K) and the ICE-generated cleavage products (28 and 17.5K) are shown. **b**, p45 is cleaved by affinity-purified ICE. Radiolabelled p45 was synthesized in a rabbit reticulocyte lysate (lane 1) and incubated with 10 units of enzyme (lanes 2–4) either alone (lane 2) or with 200 nM inhibitor B or inhibitor C (lanes 3 and 4). Cleavage was assessed by SDS-PAGE.

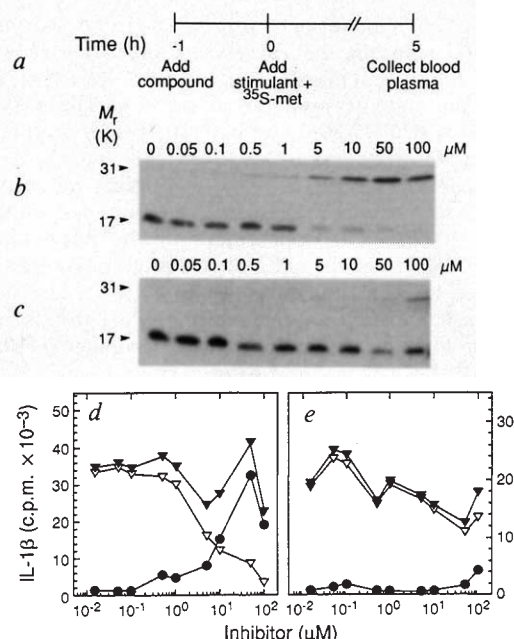
METHODS. **a**, The 1.5-kb *Eco*R1 fragment containing the full-length ICE cDNA was cloned into an expression vector under the control of the HIV 5' LTR.



ICE expression plasmid (6 μ g) and 0.6 μ g of pXSTAT were cotransfected into COS-7 cells (2×10^5 cells, 40 μ g Lipofectin) according to the manufacturer's instructions (BRL/GIBCO). Cells were collected 48 h after transfection, lysed in Triton X-100, and assayed for ICE activity^{12,24}. **b**, p45-cleavage assays were as follows: 5 μ l reticulocyte lysate programmed with T7-transcribed ICE RNA was mixed with 10 units affinity-purified ICE alone or with 200 nM of either inhibitor (18 h, 25 $^{\circ}$ C) and analysed as described¹².

FIG. 7 Inhibition of mature 17.5K IL-1 β production by whole human blood monocytes by the tetrapeptide aldehydes, inhibitor B and inhibitor C. *a*, Experimental protocol for pulse-chase experiments. *b*, *c*, SDS-PAGE of immunoprecipitated pIL-1 β (31K) and mL-1 β (17.5K) after stimulation of whole blood with heat-killed *S. aureus* (HKSA) in the presence of the indicated concentrations of inhibitor B (*b*) or inhibitor C (*c*). *d*, *e*, Plot of c.p.m. in pIL-1 β (●) and mL-1 β (▽) and the total (▼) of both from *b* and *c*, respectively.

METHODS. *a*, Heparinized (10 units ml⁻¹) human blood (1 ml) was incubated with the indicated concentration (μ M) of one of two tetrapeptide aldehydes (*b*, *d*, inhibitor B K_i = 0.76 nM; *c*, *e*, inhibitor C, K_i = 1.5 μ M), allowed to incubate for 1 h, and then supplemented with 50 μ Ci [³⁵S]methionine (1,076 Ci mmol⁻¹) and 10⁶ colony-forming units per ml. HKSA. *b*, *c*, After incubation at 37 °C, 5 h, the plasma was removed, immunoprecipitated with mono-specific rabbit anti-hIL-1 β IgG, and analysed by SDS-PAGE and fluorography. *d*, *e*, The 31 and 17.5K bands were counted using a radioanalytical imaging system (Ambis Systems) and plotted versus inhibitor concentration. The c.p.m. in the 17.5K bands were multiplied by 1.86 before plotting to correct for the loss of methionine residues on conversion of pIL-1 β to mL-1 β . Total c.p.m. = c.p.m. pIL-1 β + corrected c.p.m. mL-1 β .



Discussion

We have defined here the primary structure, molecular organization and catalytic mechanism of ICE, and established its role in the processing of pIL-1 β . The enzyme meets all chemical and catalytic criteria for a cysteine protease, but its sequence is not homologous to any known cellular cysteine protease. It is a heterodimer composed of two subunits, p20 and p10. Although p20 contains the catalytic cysteine, kinetic data provide compelling evidence that both subunits are required for catalytic activity.

The subunits, which are proteolytically derived from a single proenzyme, p45, are flanked by Asp-X bonds. This and the observation that p45 is itself an ICE substrate suggest that activation of ICE is, in part, autocatalytic. That p45 was stable after translation *in vitro* rules out an intramolecular autoprocessing mechanism and suggests that p45 is inactive. Activation of ICE presumably requires limited proteolysis by an unidentified protease, in a manner similar to prostromelysin, before autocatalysis can proceed³⁰. The detection of ICE activity in transfected COS-7 cell lysates indicates that p45 can also be activated in non-lymphoid cells.

Consistent with the catalytic mechanism and specificity of ICE, the peptide aldehyde transition state analogue, inhibitor B, is a potent, selective inhibitor (K_i = 0.76 nM). The selectivity for ICE was demonstrated by affinity chromatography, which enabled >100,000-fold purification of catalytically active enzyme to homogeneity from THP.1 cells. This inhibitor was used to demonstrate that ICE alone is necessary and sufficient for production of mL-1 β in human blood monocytes stimulated by heat-killed *S. aureus*. A commensurate increase in the plasma level of pIL-1 β accompanied inhibition, indicating that secretion of IL-1 β is not mechanistically coupled to the processing function of ICE. Furthermore, the released pIL-1 β was stable in blood and did not seem to be susceptible to partial activation by proteases, such as cathepsin G, which are capable of cleaving pIL-1 β near Asp 116-Ala 117. Nevertheless, it is possible that such 'bystander' proteases exist at sites of inflammation removed from blood³¹. This uncertainty, and the known ability of IL-1 α and tumour necrosis factor- α to mediate many of the same biological responses as IL-1 β , will require studies *in vivo* to assess fully the therapeutic potential of ICE inhibitors. □

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Superconductivity at 110 K in the infinite-layer compound $(\text{Sr}_{1-x}\text{Ca}_x)_{1-y}\text{CuO}_2$

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THE 'infinite-layer' parent structure¹ of the copper oxide superconductors (Fig. 1) is the simplest structure containing the CuO_2 sheets that are apparently essential to high-transition-temperature (high- T_c) superconductivity. At ambient pressure only $\text{Ca}_{1-x}\text{Sr}_x\text{CuO}_2$ with $x \approx 0.1$ can be stabilized in this structure^{1,2}, but at high pressures and temperatures compounds ranging from $\text{Ba}_{1/3}\text{Sr}_{2/3}\text{CuO}_2$ to $\text{Ca}_{2/3}\text{Sr}_{1/3}\text{CuO}_2$ through SrCuO_2 can be synthesized³. We have previously reported superconductivity with $T_c = 40\text{--}100\text{ K}$ in the $\text{Ba}\text{--}\text{Sr}\text{--}\text{Cu}\text{--}\text{O}$ system^{4,5}, but have not until now been able to isolate a superconducting phase. Here we report the isolation of an alkaline-earth-deficient infinite-layer phase, $(\text{Ca}_{1-x}\text{Sr}_x)_{1-y}\text{CuO}_2$ ($y \approx 0.1$), with T_c up to 110 K. In contrast to $\text{Sr}_{1-x}\text{R}_x\text{CuO}_2$ (with R a rare-earth element and $T_c \leq 43\text{ K}$), which from the composition dependence of the lattice constants is thought to be an n-type superconductor^{6,7}, our data suggest that the present superconductor is of p-type, with the carriers arising from calcium and strontium vacancies. High-resolution electron micrographs reveal defect layers, which we suggest are where the calcium and strontium vacancies are concentrated.

Samples were prepared starting from metallic compositions of $\text{Ca}_{1-x}\text{Sr}_x\text{:Cu} = 1-y:1$ with $0.4 \leq x \leq 0.7$ and $0 \leq y \leq 0.1$. Typically, a gold capsule was filled with an intimate mixture of $\text{Ca}_{0.3}\text{Sr}_{0.7}\text{CuO}_2$ (low-pressure phase) and fine powder of CuO , pressed almost isostatically up to 6 GPa using a classical cubic-anvil-type apparatus, and heated at 1,273 K with a graphite-sleeve heater under pressure for 30 min. An oxidizing atmosphere was generated by the addition to the capsule of KClO_4 , which releases oxygen at high temperature. Alternatively, a reducing atmosphere created by the graphite heater could be used if the sample powder was packed in a porous boron nitride cell. After treatment, the sample was quenched to room temperature before releasing the pressure. Before making measurements on the samples we used a scanning electron microscope equipped with an energy-dispersive X-ray analyser to check that they were free from potassium and chlorine within experimental error. It was also confirmed that the $\text{Ca}\text{:Sr}\text{:Cu}$ ratio was in agreement with the mixing ratio.

Figure 2 shows the X-ray diffraction pattern of a superconducting sample with alkaline-earth content $A = \text{Ca}_{0.3}\text{Sr}_{0.7}$ and $y = 0.1$ treated in an oxidizing atmosphere. Most peaks could be indexed on the tetragonal infinite-layer structure with $a = 3.902\text{ \AA}$ and $c = 3.350\text{ \AA}$. Small amounts of CuO and unknown phases, which probably formed as a result of slight reaction with the sample container, were present, but their relevance to superconductivity was ruled out from the absence of any positive correlation between their amount and the superconducting volume fraction. Magnetic and electrical data for this sample are shown in Fig. 3. The magnetic susceptibility (M/H , where M is magnetization and H is magnetic field) was measured for crushed sample powder on cooling in an applied field of 10 Oe, and the resistance was measured by the usual four-terminal method. The superconducting transition seems to occur in two steps, one at 110 K and the other at 75 K. The multiplicity has been attributed to a specific microstructure to be described later. A superconducting volume fraction of $\sim 10\%$ was estimated at 5 K. This is reasonably large for the fine particle size of $\leq 1\text{ }\mu\text{m}$ measured by scanning electron microscopy. Resistance begins to drop at $\sim 110\text{ K}$ and becomes zero within experimental error at 35 K.

We did not find any substantial difference between the X-ray diffraction patterns of the superconducting sample and the stoichiometric insulator³ except for a slight peak broadening in the former. The broadening is index-dependent, slight for $(hk0)$ peaks and serious for (00ℓ) peaks. In agreement with this,

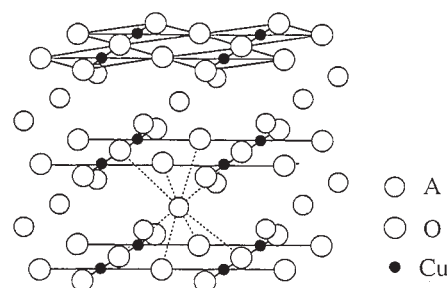


FIG. 1 Infinite-layer structure.

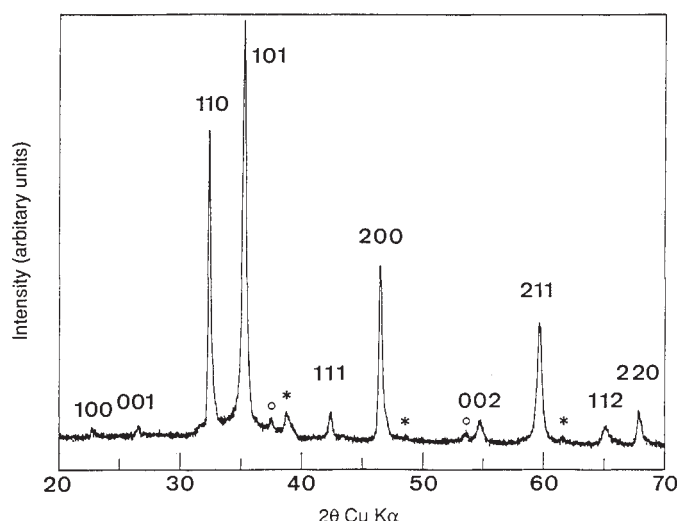


FIG. 2 X-ray diffraction pattern of $(\text{Ca}_{0.3}\text{Sr}_{0.7})_{0.9}\text{CuO}_2$. Peaks attributable to CuO are marked by asterisks, unknown phases by open circles.

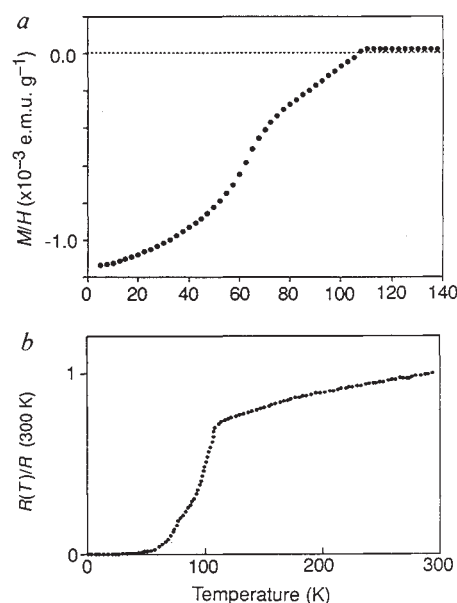


FIG. 3 a, Temperature dependence of susceptibility (M/H) measured for a powdered sample with $A = \text{Ca}_{0.3}\text{Sr}_{0.7}$ and $y = 0.1$ on cooling in an external field of 10 Oe. b, Temperature dependence of resistance of a sintered pellet with the same composition measured by the four-terminal method.

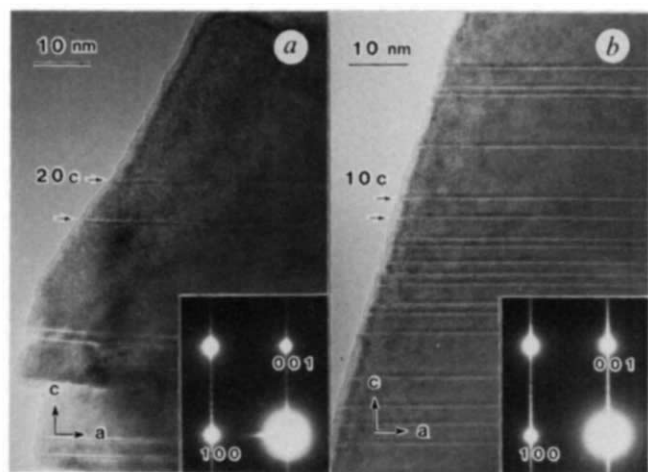


FIG. 4 Electron diffraction patterns and the corresponding high-resolution electron microscope images of samples $A=\text{Ca}_{0.3}\text{Sr}_{0.7}$, $y=0$ (a) and $A=\text{Ca}_{0.4}\text{Sr}_{0.6}$, $y=0.1$ (b).

electron diffraction spots are streaked along the c^* axis (insets, Fig. 4). High-resolution electron microscopy reveals the origin of the broadening and streaking (Fig. 4): 'defect' layers are inserted in the structure in a fairly random way. Such defects are clearly related to the deficiency of A atoms rather than to changes in oxygen content, because the density of the defect layers decreases as the A:Cu ratio approaches unity. Figure 4a, an image of a sample for which $y=0$, shows fewer defect layers than Fig. 4b, for which $y=0.1$. Moreover, essentially the same microstructure was found for $(\text{Ca}_{0.3}\text{Sr}_{0.7})_{0.9}\text{CuO}_{2-\delta}$ ($\delta > 0$) prepared in an inert or reducing atmosphere, although its physical properties depended strongly on the atmosphere. We therefore consider that A-ion vacancies are not distributed randomly but tend to be concentrated in the defect layers. The reason why even a sample with a starting composition of $y=0$ is slightly defective is that the temperature of the high-pressure treatment is high enough to drive the system towards a slightly A-deficient composition⁵.

At higher magnification, two important features of the defect layer are seen (Z.H., M.A., M.T. and Y.B., manuscript in preparation). The triple Cu-O sheets involved in each defect layer have an elongated intersheet distance of ~ 3.7 Å, 10% longer than the normal distance, and the contrast of the oxygen sites within the central Cu-O sheet changes, depending strongly on the atmosphere during high-pressure treatment. Oxygen released from KClO_4 can fill the oxygen vacancies within the central sheet which are stabilized in an inert or reducing atmosphere. It is reasonable to assume that the distance between CuO_2 sheets is elongated if the A-ion vacancies are concentrated in the defect layer, as the deficiency enhances the electrostatic repulsion between the negatively charged CuO_2 sheets.

It might be suspected that a structural block containing pyramidally coordinated Cu ions, like the $[\text{Cu}(2)\text{O}_2/\text{AO}/\text{Cu}(1)\text{O}/\text{AO}/\text{Cu}(2)\text{O}_2]$ block in $\text{YBa}_2\text{Cu}_3\text{O}_7$ (where A is Ba) and in $\text{Nd}_2\text{Ce}_{2/3}(\text{Ba}, \text{Sr})_{4/3}\text{Cu}_3\text{O}_9$ (A is $\text{Nd}_{1/3}(\text{Ba}, \text{Sr})_{2/3}$) (ref. 9) forms also in the present superconductor when prepared in an oxidizing atmosphere. We consider this unlikely because the thickness of these oxygen-rich blocks is > 8 Å (refs 8, 9), much longer than the value of ≤ 7.4 Å observed in the present phase; moreover, as mentioned above, the defect layers arise from the alkaline-earth deficiency, irrespective of oxygen content. We therefore formulate the defect layer as $\text{CuO}_2/\text{A}_{1-z}/\text{CuO}_{2-\delta}/\text{A}_{1-z}/\text{CuO}_2$ ($y < z$), where $\delta (\geq 0)$ depends on the atmosphere. The superconductivity appearing at $\delta \approx 0$ must be of p-type, with carrier holes created by the A-deficiency. The superconducting volume fraction tends to increase with

increasing A-deficiency (that is, with increasing defect-layer density) for samples treated in an oxidizing atmosphere. The two superconducting transitions thus probably arise from inhomogeneity in the defect-layer density within a sample. Finally, we note that $\text{A}_{1-z}\text{CuO}_2$ is superconducting throughout the wide range of A mentioned above, but that T_c remains constant at 110 K. □

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Pentagons, heptagons and negative curvature in graphite microtubule growth

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THE standard carbon-arc synthesis for fullerenes also produces graphitic microtubules with helical structures¹. In most cases the cylindrical tubes are closed by polyhedral caps, some being first transformed into a conical shape before closure². Here we present images from transmission electron microscopy of a further kind of growth morphology, in which cone-like growth is transformed into cylindrical growth by the incorporation of a defect that induces negative curvature. We suggest that the defect in the hexagonal network responsible for negative curvature may be a single heptagonal ring. Three-dimensional, open negatively curved graphitic structures have been proposed recently by Terrones and Mackay³. We discuss more generally the effect of pentagons and heptagons on the growth morphologies of these tubules, and the constraints on the number of pentagonal defects imposed by tube closure.

Graphitic microtubules are formed in the carbon deposit in a carbon arc. We find that the deposit is composed mainly of pyrolytic graphite with no amorphous carbon, as the high reaction temperature (over 2,500 °C) in the arc favours graphitization. The tubules are usually attached to the pyrolytic graphite at one end, the other end being free. This suggests that the tubules grow at their free ends, as is common in whisker growth. The tubules are not always cylindrical; we have seen many variations in shape, especially near the tube tips, and have classified the morphologies into several groups. Polyhedral graphitic particles coexist with the tubules in the carbon deposits. The particles are thought to be formed as a consequence of homogeneous growth in all directions, whereas the tubes grow in one direction because of a helical structure in the carbon hexagonal network¹. The growth nuclei controlling the growth morphologies of the graphitic particles have not yet been understood.

Among many tube-tip shapes, one that we repeatedly observed in the transmission electron microscope (TEM) is represented in Fig. 1. The tubule, hollow at the centre, comprises three portions: on the right, a nanometre-sized tube of five graphitic cylinders with its tube-tip closed by polyhedral surfaces; in the

FIG. 1 Electron micrograph showing recurrent terminations of graphitic microtubules consisting of three portions: a thin cylindrical tube (right), a cone (middle) and a thicker cylindrical tube (left). Individual dark lines (lattice images) correspond to graphitic sheets separated by 0.34 nm. The internal cone are often closed in pairs. Regions indicated by A and B contain disclinations.

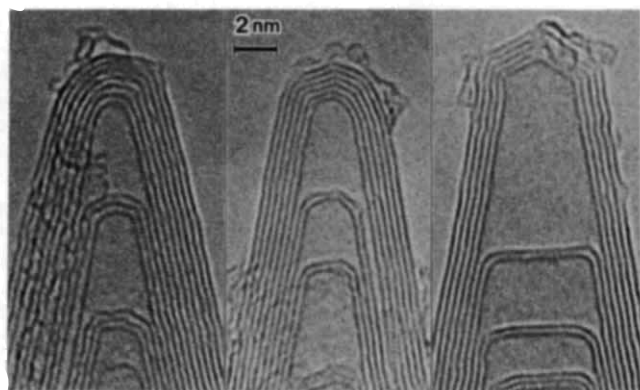
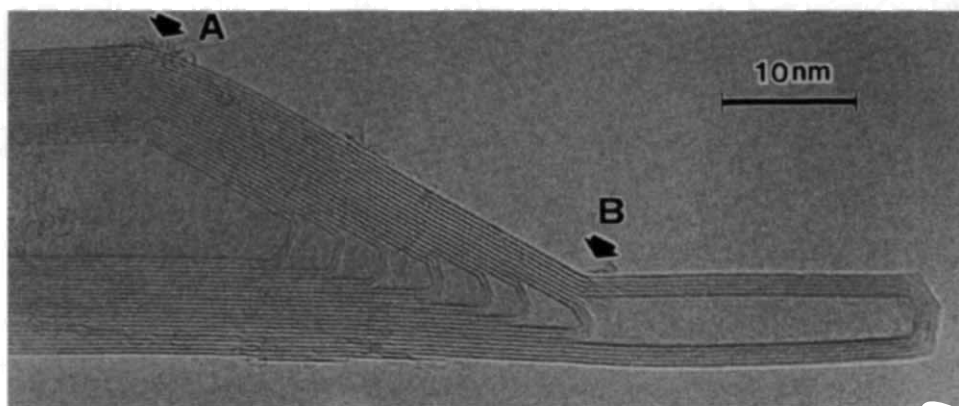


FIG. 2 Terminations with identical conical caps at the tube-tips. Some caps are faceted. The cone angles are roughly 20° , close to that for a cone made by connecting two sides of an equilateral triangle. Shells made up of two or three graphitic sheets are stacked like ice-cream cones. We believe that this is the final form in the cone growth. The caps of individual cones are expected to have five pentagons according to Euler's theorem.

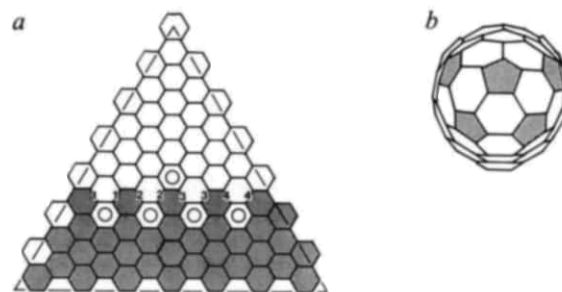


FIG. 3 *a*, The 20° cone shape of Fig. 2 can be reproduced by joining two triangle edges of this carbon hexagon network. *b*, Model for the smallest 20° cone tip, viewed along the cone axis, which has mirror symmetry with five pentagons. The structure is formed by connecting shaded hexagon edges labelled with the same members in *a*. Circles indicate positions where the pentagons are formed.

middle a cone with stacked 'ice-cream cone' terminations of inner graphitic shells; and on the left, a wider tube which extends towards the left.

We frequently observed nearly regular cone-like terminations which were more common in thicker tubes. Cone angles seen in TEM images (Fig. 2) are typically $\sim 20^\circ$, varying over the range $15\text{--}30^\circ$ (refs 1, 2). The angle corresponds to that of a cone made by connecting two sides of a regular triangle as illustrated in Fig. 3*a*. The hexagon sheet is perfectly continuous all around the cone surface. To close the apex, five pentagons are required in the positions indicated by open circles; the closed cone is shown in Fig. 3*b*. In general, the cones observed are 3–5 times larger than this example. A similar cone shape has been observed in graphitic carbons prepared by thermal decomposition of aromatic hydrocarbons (M. Endo and H. Kroto, personal communication). They thought that the cone at the tube-tip was always closed but could grow by breaking some of the carbon bonds.

The (002) lattice fringes appearing near the tube-tips at bottom of Fig. 1 run horizontally through all three sections (the thick tube, cone and thin tube). This means that the graphitic sheets in this side of the tube consist of perfectly continuous hexagon networks. Where the top side of the tube forms a tapered step (middle portion), the lattice fringes run parallel to this step. The number of lattice fringes, and thus the wall thickness of the tube, vary from location to location but are always the same at both sides of the central hollow. This also applies to the cones shown in Fig. 2. The presence of equal numbers of fringes is due to the fact that the individual graphite sheets forming the multi-shell structure are perfectly continuous even around the

transition region (across the cone). It is sufficient, therefore, to consider only the outermost shell in the following discussion, as each shell is more or less the same shape.

An immediate question about the tube shape is how the small and large cylinders are connected topologically to the ends of the cone without disturbing the continuity of the graphite sheets.

Considering the characteristic morphologies, we constructed a topological model in which pentagons and heptagons play a key role in the tube-tip shapes. An apex such as that indicated by arrow A in Fig. 1 must incorporate a single pentagon into a hexagon sheet as illustrated in Fig. 4*a*. The illustration is a projection of the topological surface on the plane of the page. The network, which has 5-fold symmetry, can be obtained by removing the shaded hexagons in the regular triangle shown in Fig. 4*b*. The structure proposed here is a kind of defect in a topological surface, described as a $+60^\circ$ wedge disclination^{4,5}. A surface containing a pentagon is curved positively. The topological surface transforming the tube into a cone can be achieved by superimposing the hexagon edges indicated by heavy lines at both sides of the network in Fig. 4*a*. The shaded hexagons form the cone shape, and the remaining hexagons are responsible for the tube. In the tube structure, the ideal 5-fold symmetry about the pentagon is lost and mirror symmetry appears about a vertical line. The mirror symmetry, however, will also be destroyed in most tubes, as they usually have a helical (and chiral) structure. A helical tube can be formed by joining two sides of the network shown in Fig. 4*b* after sliding one side up or down with respect to the other. Introduction of a pentagon into a hexagon network on the cylinder will result in a strain around the pentagon. This strain effect is noticeable as a slight

distortion to the cylinder at A in Fig. 1, indicating that the cross-section of the tube at A is not perfectly circular but oval.

The opposite situation occurs at the point indicated by arrow B, where the graphitic shell is folded back to lie in the same direction as in the thicker tube. The hexagon network of this region was obtained by introducing a -60° wedge disclination, in which a single heptagon is formed (Fig. 4c). Insertion of an extra regular triangle produces a negative surface^{4,5}. The network has 7-fold symmetry, but topologically it has a saddle shape with mirror symmetry about a vertical line. Mackay and Terrones³, and Lenosky and others^{6,7}, have pointed out the role of seven- or eight-membered rings in forming periodic minimal surfaces from carbon hexagon networks.

The three-dimensional shape corresponding to portion B in Fig. 1 can be obtained by rolling back together the two sides of the hexagon network indicated by heavy lines in Fig. 4c. The presence of both a $+60^\circ$ disclination and a -60° disclination on a closed hexagon surface restores the original shape, in this case a cylinder. A combination of two $+60^\circ$ disclinations and one -60° disclination results in the equivalent to a single $+60^\circ$ disclination (Fig. 4d), which is another possible candidate for the defect at A in Fig. 1. Euler's theorem leads to a relation $P = S + 12$, where P and S are the numbers of pentagons and heptagons incorporated into a closed hexagonal network. For $S = 1$, we have $P = 13$. In other words, the number of pentagons should always exceed the numbers of heptagons by 12 in any closed surface of the hexagonal network. Such a surface will contain negative curvature. Very complicated morphologies of graphitic spheroidal particles may be caused by these pentagons and heptagons and their combinations⁸.

In short, the occurrence of a single pentagon at the open end of a tube leads inevitably to a cone shape, and eventually the

cone tip will become closed as illustrated in Fig. 3b, unless the formation of a single heptagon on the cone restores a cylinder. We conclude that the tube can continue to grow as long as only hexagons are being formed on the open end of the tube. Formation of a hexagon requires two carbon atoms to be added at each kink site on the periphery, whereas a single atom is required for the pentagon. One possible reason for the formation of pentagons seems to be a drop in carbon supply during carbon-arc evaporation. In the normal evaporation experiment, the arc is fairly unstable and typically lasts for several tens of seconds, after which the carbon-arc electrodes must be realigned for further evaporation. We believe that flux density of carbon atoms decreases towards the end of the arc discharge, and as soon as the arc stops, the tube-tips are closed quickly by forming pentagons. In contrast, a higher flux of carbon atoms may increase the rate of formation of heptagons, which need three carbon atoms at each kink site.

Once a cap is formed on the tip of a cylinder tube, it can grow no longer. Capping the cylinder tube tips should be completed by clustering six pentagons near the tube tips according to Euler's theorem. The apices of the polyhedral shapes making up the tube tips (Fig. 1), cone tips (Fig. 2) and many spheroidal particles^{8,9} are very probably caused by pentagons. To cap a cone-tip, five pentagons will be sufficient because one pentagon has already been used for the $+60^\circ$ disclination. Variations in the polyhedral shapes reflect many possible distributions of the pentagons on the tube tips, unlike the more symmetrical distribution of pentagons in the truncated icosahedron of C_{60} . □

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Rough-flat-rough transition of crystal surfaces

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ABOVE a characteristic temperature, the free energy required to form a step on the surface of a growing crystal may fall to zero; the surface then undergoes a transition from smooth (faceted) to irregular (rough) growth. Here we describe studies of the growth of $n\text{-C}_{21}\text{H}_{44}$ crystals from n -hexane solutions close to the roughening transition. Beginning with equilibrium crystal growth at a temperature slightly above the roughening transition, we introduce progressively greater supersaturations by lowering the temperature. Observations of the growth morphology reveal a transition from rough to smooth growth, followed at greater supersaturations by a transition back to rough growth. To our knowledge, such a sequence of rough-smooth-rough crystal growth has not been reported before. Our measurements of the step free energy of the $\{110\}$ faces indicate that it vanishes discontinuously at the equilibrium roughening transition temperature, in contrast to the continuous (critical) behaviour predicted by theoretical models^{1–11}.

Since Burton, Cabrera and Frank^{1,2} first applied the results of the theory³ of order-disorder phase transition in a two-dimensional Ising lattice to crystal surfaces, the roughening phase transition and kinetic roughening have been studied in

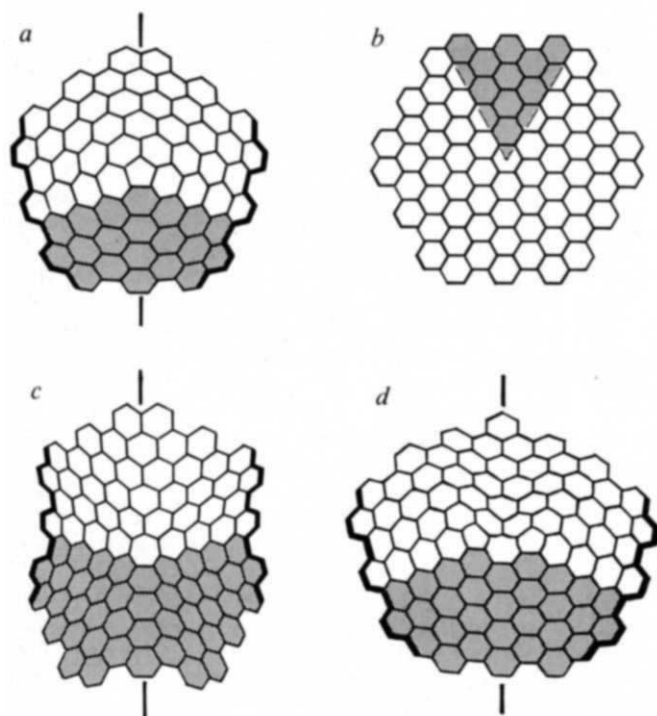


FIG. 4 a, A $+60^\circ$ disclination produced by introducing a pentagon into a hexagon network. This takes place at position A in Fig. 1. A three-dimensional network including the portion A can be reproduced by joining the two sides indicated by heavy lines. Shaded hexagons form the 20° cone and the rest form a tube. b, The $+60^\circ$ disclination is formed by removing shaded hexagons. c, A -60° disclination produced by including a heptagon, to form a saddle-shaped surface (negative curvature) like that at B in Fig. 1. Its three-dimensional structure can be obtained by joining hexagon edges drawn in heavy lines. d, Complex disclination, consisting of one heptagon and two pentagons, which is equivalent to the single $+60^\circ$ disclination in a.

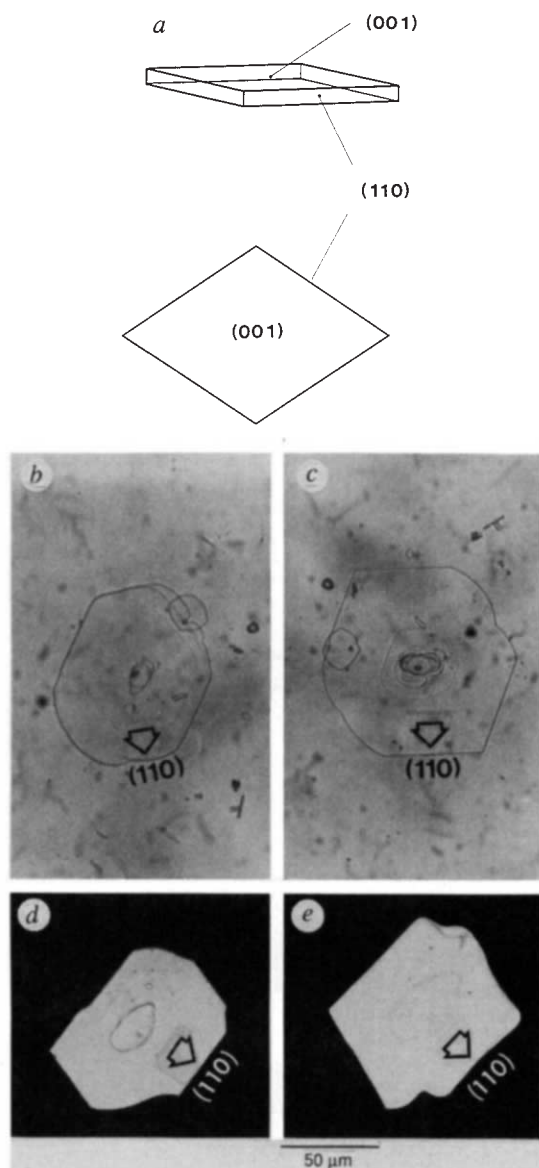


FIG. 1 a, Theoretical morphology of odd-numbered paraffins. b–e, Morphological transitions of a crystal of $n\text{-C}_{21}\text{H}_{44}$ growing from n -hexane solution ($X = 2.93 \times 10^{-2}$, where X is mole fraction of solute, $T_s \approx 0.14^\circ\text{C}$ ($T_s \geq T_{110}^*$)). b, Rounded {110} faces at low supersaturation; $\sigma = 0.3264\%$, $\sigma < \sigma_1^c$. c, d, Kinetic faceting of {110} faces; $\sigma = 0.7618$ and 1.306% respectively, $\sigma_2^c > \sigma > \sigma_1^c$. e, Kinetic roughening of {110} faces; $\sigma = 2.287\%$, $\sigma > \sigma_1^c \approx 0.435\%$, $\sigma_2^c \approx 1.524\%$. b, c, Bright-field microscopy; d, e, cross-polarizing microscopy.

great detail^{4–28}. Monte Carlo simulations^{12–16} led theoreticians to characterize the roughening transition as continuous transition of infinite order ('Kosterlitz–Thouless' roughening^{8–11}). Roughening phenomena have also been observed experimentally on crystal faces^{17–25}. According to the solid-on-solid (or SOS) lattice model^{4,5} if the temperature approaches the roughening temperature T_r from below, the order parameter (the edge free energy per structural unit, γkT , where k is the Boltzmann constant and T the temperature) vanishes continuously as

$$\gamma \sim \exp[-\alpha(T_r - T)^{-1/2}], \quad T \leq T_r \quad (1)$$

(α is a coefficient depending on the system). The implication of equation (1) is that at $T_s < T_r$ (T_s denotes equilibrium temperature) a two-dimensional nucleation barrier exists because $\gamma > 0$. Then we have a flat surface at equilibrium or at a small supersaturation σ ($\sigma = \Delta\mu/RT$, where $\Delta\mu$ is the chemical poten-

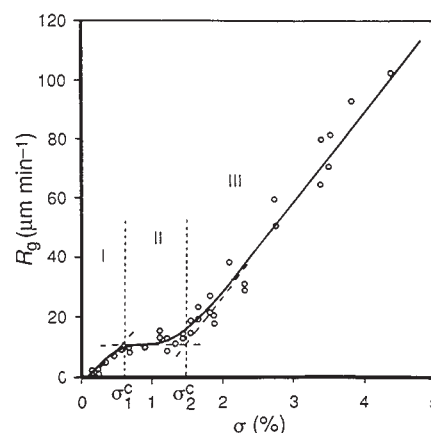


FIG. 2 Growth rate R_g of the {110} faces of $n\text{-C}_{21}\text{H}_{44}$ growth from n -hexane solutions. $X = 2.939 \times 10^{-2}$, $T_s \approx 0.16^\circ\text{C}$, $T_s > T_{110}^*$, $\sigma_1^c \approx 0.72\%$, $\sigma_2^c \approx 1.52\%$. The growth-rate curve is divided into three areas (I, II and III) corresponding to the two normal cases ($T > T_r$, area I; $T < T_r$, areas II and III). In area I the linear part starts from the origin, implying no nucleation barrier in the growth process. In areas II and III the kinetics of growth show that at a critical value σ_1^c the original rough face changes into a flat face, which will grow by a layer-by-layer mechanism; at $\sigma > \sigma_2^c$, the growth curve becomes linear again, because of kinetic roughening.

tial difference between the solid and the liquid phase and R the gas constant). In this case only two-dimensional nucleation or spiral growth is possible, and the growth rate R_g is a nonlinear function of σ for $\sigma < \sigma^c$ (σ^c is a critical supersaturation)^{26,27}. When $\sigma \geq \sigma^c$, the nucleation barrier vanishes in the supersaturation environment, and 'kinetic roughening' occurs. The originally flat faces roughen kinetically, and the relation between R_g and σ becomes linear^{27,28}. A relation between edge free energy and critical supersaturation for circular nuclei²⁸ can be expressed as

$$\sigma^c = \pi\gamma^2 \quad (2)$$

At $T_s \geq T_r$, $\gamma = 0$, and the interface therefore becomes rough on the molecular scale and macroscopically rounded; hence, the crystallographic orientation of the surface is lost. This corresponds to the (thermal) roughening transition. It follows that R_g is linearly dependent on the supersaturation σ .

Here we describe novel roughening phenomena occurring at the {110} surfaces of n -paraffin crystals in n -hexane solutions, from which we derive the behaviour of the order parameter γ of this system close to T_r . The n -paraffins used in our experiments are from analytically pure chemicals (Alfa, >99.0%) and the n -hexane is spectroscopically pure (March, >98.0%). To obtain a well defined supersaturation in solutions of fixed composition, as usual we cool the solution from T_s to a certain value. From the definition of supersaturation, it follows that for a regular solution the supersaturation is linearly proportional to the supercooling ΔT

$$\sigma \approx \frac{\Delta h^{\text{diss}}}{RT_s^2} \Delta T \quad (3)$$

(Here Δh^{diss} is the molar dissolution enthalpy and $\Delta T = T_s - T$). For further details of experiments, see refs 29, 30.

Crystals of n -paraffin may exist in one of four forms³¹. Here we focus our attention on odd-numbered n -paraffins which are orthorhombic with space group P_{bcm} (ref. 32). Theoretical analysis^{30,33} indicates that crystals of odd n -paraffins are lozenge-shaped (Fig. 1a). We chose the {110} faces of crystals of $n\text{-C}_{21}\text{H}_{44}$ for our investigations.

We explore the situation where the equilibrium temperature is slightly higher than the roughening temperature ($0 < (T_s - T_r) < 0.1^\circ\text{C}$), and introduce supersaturations by lowering the temperature. At equilibrium or a small supersaturation, the

interface is rough: no facets in the $\{110\}$ orientation develop on the growth form (Fig. 1b). If the supersaturation exceeds a critical value σ_1^c , however, $\{110\}$ facets develop (Fig. 1c). As the supersaturation increases further, exceeding another critical value σ_2^c , the faceted faces roughen kinetically (Fig. 1d, e). The two transitions are completely reproducible. In the reverse process, exactly the same values σ_2^c and σ_1^c are obtained by lowering σ from above σ_2^c . In this 'rough-flat-rough' sequence, we consider rough-to-smooth transition occurring at σ_1^c to be the most important, because it occurs at a temperature very close to the thermal roughening transition temperature and, to our knowledge, has not been observed before. This faceting, developing only in a supersaturated environment, may be called 'kinetic faceting'. It is different from the normal faceting which may occur at equilibrium.

To clarify the 'rough-flat-rough' transition, we determined the growth rates of $\{110\}$ faces as a function of supersaturation σ . Figure 2 shows a typical example of the process ($T_s \geq T_r$). The growth-rate curve is divided into three areas which are a combination of the two typical normal cases: $T_s > T_r$ (area I) and $T_s < T_r$ (areas II and III). The kinetic data clearly demonstrate that the development of facets on the rough faces is because the faces regain edge free energy ($\gamma > 0$) at a certain supersaturation. This strongly supports our observations presented above. The two critical values of σ_1^c and σ_2^c can be determined by extrapolating the linear parts of the curve to cut the horizontal line crossing the point of inflexion or independently by carefully observing the morphological transition^{29,30}. The results obtained by the two methods agree within experimental error. In our experiments we mainly used the second method, which offers an accuracy of $|\Delta\sigma| < 0.1\%$.

We know of no model that can directly explain our observations on this complicated organic system. If we assume that the SOS Kossel model is valid to some extent for the n -paraffin system, we can combine equations (1) and (2) and plot the σ^c curve, together with the trajectories (based on equation (3)) of the system in the phase space of (σ , T) starting from different T_s values (see Fig. 3). It can be seen from the schematic diagram that the phase space of (σ , T) is divided into two regimes by the σ^c curve. If crystal growth takes place under the σ^c curve, faceted growth is expected; above the σ^c curve, rough growth is expected. We now focus on dashed line c, which starts from a temperature T_s slightly above T_r , and cuts the σ^c curve at two points (σ_1^c and σ_2^c). At $\sigma_{\text{system}} < \sigma_1^c$, the growth of crystal faces

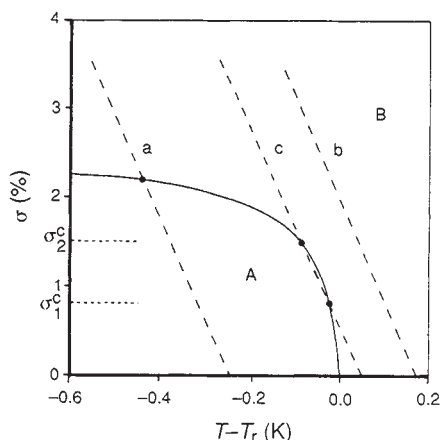


FIG. 3 Schematic diagram of the phase space of (σ , T). The solid line is the σ^c curve obtained from $\sigma^c = C \exp[-\alpha'(T_r - T)^{-1/2}]$ by combining equations (1) and (2) ($C = 2.55 \times 10^{-2}$, $\alpha' = 0.150$); the dashed lines, based on equation (3) ($\Delta h^{\text{diss}} = 6.411 \text{ kcal mol}^{-1}$), represent trajectories of the system starting from different values of T_s . The phase space is divided by the σ^c curve into two regimes: A, flat interfaces; B, rough interfaces. Lines a and b represent two normal cases ($T_s < T_r$ and $T_s > T_r$, respectively). Line c corresponds to the case of T_s slightly above T_r .

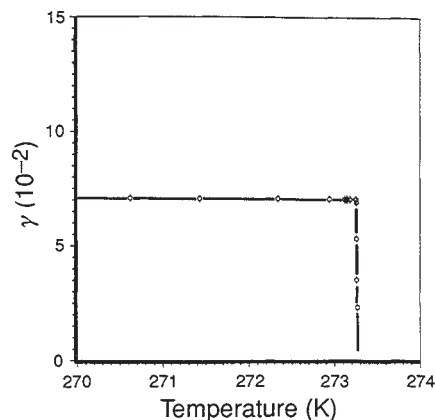


FIG. 4 Edge free energy γ and temperature T for the $\{110\}$ faces of $n\text{-C}_{22}\text{H}_{44}$ crystals in n -hexane solutions. $T_{110}(21) = 273.27 \pm 0.03 \text{ K}$.

is within the rough regime. As soon as $\sigma_{\text{system}} \geq \sigma_1^c$, the growth re-enters the flat regime. At that point, 'kinetic faceting' occurs. As σ_{system} continues to increase to above σ_2^c , growth enters the rough regime again, resulting in kinetic roughening of the faces.

From equation (2) and the values of σ^c determined from our experiments, we obtained a curve of γ against temperature for the $\{110\}$ faces of $n\text{-C}_{21}\text{H}_{44}$ crystals in n -hexane solutions (Fig. 4). Rather surprisingly, the $\gamma(T)$ curve has the shape of a step function. This result strongly suggests that the roughening transition in the $n\text{-C}_{21}\text{H}_{44}/n$ -hexane system is much closer to a first-order phase transition than to an infinite-order or second-order (the order-disorder) phase transition. The occurrence of this kind of roughening transition may be due to a strong coupling of the thermal roughening with a structural surface phase transition related to a change in the rotation states of the molecules. Work is in progress to investigate this. □

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Climate-driven pH control of remote alpine lakes and effects of acid deposition

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DESPITE the attention given in recent years to the issue of lake acidification by anthropogenic emissions, the question of how climate change might influence the acid-base equilibria of lakes has been little explored. Here we use palaeolimnological data to show that the acidity of two soft-water, high-altitude lakes in the central Alps was correlated with regional temperature during the entire nineteenth century, colder years being associated with lower pH. Our findings of high concentrations of organic matter and elevated Fe/Mn ratios in the corresponding sediment layers support the view that this relationship is dictated primarily by the temperature dependence of in-lake processes of alkalinity generation. The onset of anthropogenically derived acid precipitation at the beginning of the present century led to a breakdown of the pH-temperature relationship, with pH dropping steadily to values of 5.6–5.8. Nevertheless, temperature maxima around 1950 still coincide with peaks in pH, and some pH minima before 1900 fell as low as the levels around 1970. It is an open question whether the very recent levelling off that we observe in the decline of pH is due to rising temperatures or to decreasing precipitation acidity.

Rassas See is situated at 2,682 m above sea level (m a.s.l.) in the upper Vinschgau, South Tyrol, Italy, near the junction of the Swiss and Austrian borders. Portles See is 2,892 m a.s.l. about 20 km east of Rassas. The bedrock of the catchments consists of granites and gneisses of the Oetztal crystalline. In the autumn of 1990, sediment cores of 30 cm were taken and cut into 0.5-cm slices. From each segment, we used 1 cm³ fresh material for chemical, diatom and pollen analyses¹. The pH was inferred by means of weighted averaging² after calibration with a data set comprising 31 high-altitude lakes from the Alps³. In Rassas See, the diatom distribution showed a dominance of various *Aulacoseira* species between depths of 5 and 30 cm. *Aulacoseira alpigena* var. *helvetica* and *A. valida* appeared in the oldest stratum (21–30 cm), the next (10–17 cm) was dominated by *A. lirata* and the third (5–10 cm) by *A. pfaffiana* and *A. distans*. In the section from 0 to 5 cm, *Achnanthes* species (*A. helvetica* var. *alpina*, *A. scotica*, *A. marginulata*, *A. c.f. saccula*) predominated; *Eunotia exigua* also appeared. Inferred pH values (Fig. 1) ranged from 6.51 (28.0–28.5 cm, about 1820) to 5.79 (1.5–2.0 cm, about 1980, and 19.0–19.5 cm, about 1880). The inferred pH of the top layer (pH 5.82) corresponded well with the pH measured in the lake water (pH 5.95). In Portles See, *Achnanthes* (*A. helvetica*, *A. helvetica* var. *minor* and var. *alpina*, *A. scotica*, *A. marginulata*) was the dominant diatom genus down-core. The inferred pH (Fig. 1) ranged from 5.60 (3.0–3.5 cm, about 1970) to 6.31 (19.5–20.0 cm, about 1870). Since the beginning of the twentieth century, the pH has decreased gradually by roughly 0.5 units in both lakes, and we attribute this to the effect of acid rain. Acidification of sensitive lakes in the Alps by atmospheric deposition has been reported for roughly a decade, although it has been lower than in southern Scandinavia or southern Germany^{4–6}. High-altitude stations (2,500 to 3,200 m a.s.l.) and inner alpine valleys are affected by precipitation with mineral acidity of ~50 microequivalents per litre, thus exceeding the critical load in very sensitive watersheds^{7,8}. Until now, however, palaeolimnological investigations in the Alps have shown only small changes in the pH history of high mountain lakes, except in Schwarzsee ob Sölden (Tyrol, Aus-

tria)⁹ where a pH shift from 6 to 5 at sediment depths of 35–25 cm has been documented.

Before 1910, pH values showed strong but short-term fluctuations with minima slightly above the recent values (pH 5.6–5.8). This early phase correlated with Swiss and Austrian temperature curves (Fig. 1). The ratio between timber-line arboreal pollen, consisting of *Pinus cembra*, *Larix decidua* and *Picea*, and non-arboreal pollen has generally decreased since the beginning of the nineteenth century, but it shows depressions around 1810, 1850 and 1900 which compare well with temperature minima (Fig. 1a). A first-order linear regression of pH and the mean Austrian temperature (*T*) in the period 1807–1907 was highly significant ($\text{pH} = 6.37 + 0.417T$, $n = 30$, $r = 0.685$, $P < 0.001$,

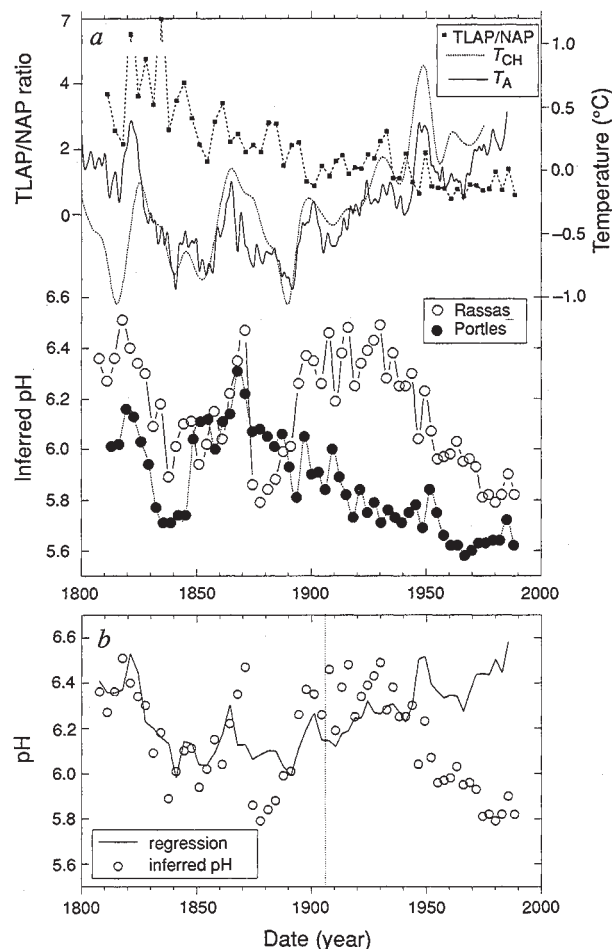


FIG. 1 a, Ratio of timber-line arboreal pollen (TLAP, consisting of *Pinus cembra*, *Larix decidua* and *Picea*) to non-arboreal pollen (NAP) in the sediment of Rassas See; Swiss temperature curve (T_{CH}) and Austrian temperature curve (T_A); and pH inferred from diatom assemblages in the sediment of Rassas and Portles See. b, Inferred pH values of Rassas See and pH calculated from a linear regression between pH and Austrian temperatures in the period 1807–1907 ($\text{pH} = 6.37 + 0.42T$, $n = 30$, $r = 0.685$, $P < 0.001$). Swiss temperatures are smoothed averages ($n = 11$) of the deviation from the mean in Basel (1901–1960)¹⁰. Austrian temperatures (20 stations) are the running averages ($n = 9$) of the deviation from the mean of the years 1951–1980 (ref. 26). The pH was reconstructed from the frustules of benthic diatoms by using the weighted average pH of up to 51 species (making up 85–98% of all taxa). They were calibrated by a data set of 31 high-altitude lakes^{3,9} in the western and central Alps ($r = 0.981$). Rassas sediments were dated by ¹³⁷Cs and ²¹⁰Pb, which both gave average sedimentation rates of 1.7 mm yr⁻¹. The sediments of Portles See were dated by ¹³⁷Cs, yielding an average value of 1.6 mm yr⁻¹. As we found only small compaction, we assumed that the sedimentation rates of the upper half of the core were 10% higher than those of the lower half. Depressions in the TLAP/NAP ratio, indicating timberline migrations depending on climate changes, correspond well with temperature minima, and thus confirm the results of ¹³⁷Cs and ²¹⁰Pb dating.

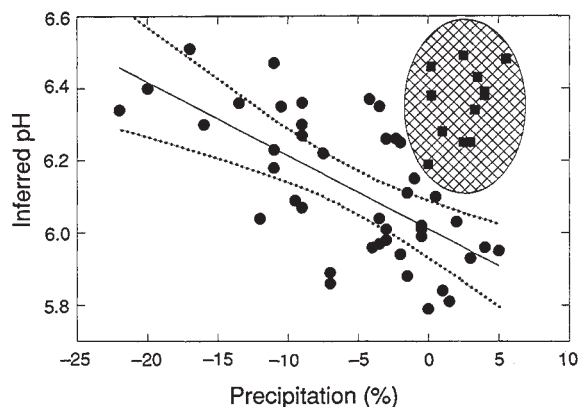


FIG. 2 Linear regression of inferred pH of Rassas See and Swiss precipitation values, p (difference from the average in Basel, 1901–1960)¹⁰ in the period 1807–1975. Crosshatched area indicates results from the period when only one species, *A. lirata*, dominated (40–80%). These points were excluded from regression analysis; $pH = 6.01 - 0.020p$, $n = 44$, $r = 0.663$, $P < 0.001$.

where T is in °C). During this period there was a significant inverse relationship between pH and precipitation ($r = -0.658$, $n = 30$, $P < 0.001$). A multiple regression between pH, temperature and precipitation (p)¹⁰ yielded a correlation coefficient of $r = 0.714$ ($n = 30$, $P < 0.001$, $pH = 6.21 + 0.27T - 0.01p$). Precipitation correlates inversely with pH up to the present day (Fig. 2). Warmer years, which are normally drier, are characterized by increased evaporation, longer residence times, more stable stratification, higher biomass production and stronger reduction processes at the sediment–water interface, thus leading to higher pH values in lake water. During cold periods, the residence times, evaporation, stratification stability and water temperatures decrease and the formation of perennial snow fields in the watershed can contribute to the discharge of unbuffered or acidic meltwater during summer. Consequently, the intensity of chemical and biological processes producing alkalinity is strongly reduced^{11,12}.

The debate on the relative importance of terrestrial and in-lake generation of alkalinity¹¹ in high-altitude lakes is still continuing^{13–15}. Our assumption that pH is controlled by climate (primarily through production and decomposition processes in the lake) is supported by the curves for organic matter (determined as loss on ignition and the Fe/Mn ratio (Fig. 3) from Rassas See which show three peaks corresponding roughly to the temperature peaks of 1820, 1860 and 1900. The close relationship between loss of ignition and the Fe/Mn ratio (Fig. 3, inset) is a strong indication that organic matter production influences the redox potential and consequently the acid–base equilibrium. Energy-dispersive X-ray analysis of the upper 18 cm showed the occurrence of iron oxides and hydroxides only in the period from 1915 to 1990; they were absent from 13.5 cm down-core, before 1915 (S. Forti, personal communication). Furthermore, the appearance of *Pediastrum* and high abundances of *Sparganium* around 1860 and 1820 support the view of a temperature-driven pH shift caused by changing redox conditions and higher productivity. The small ratio of catchment to surface area (~9.5) of Rassas See also indicates the relative importance of in-lake processes. The rise in algal productivity can counterbalance the pH increase by enhancing CO_2 saturation during decomposition of organic matter. Higher CO_2 concentrations in the water column and at the sediment–water interface, on the other hand, enhance the relative importance of in-lake alkalinity generation¹⁶, although they lead to larger seasonal fluctuations in pH (ref. 5).

The onset of acid rain in Europe¹⁷ at the beginning of the twentieth century has led to a progressive decoupling of climate and biogeochemical processes (Fig. 1a, b). In Rassas See, the

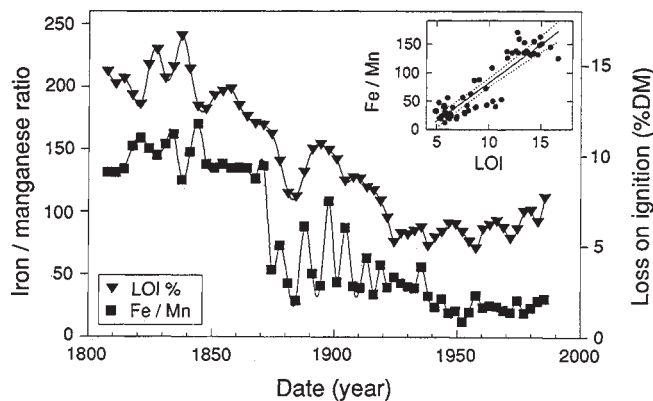


FIG. 3 Loss on ignition (LOI) and iron/manganese ratio in the sediment of Rassas See. LOI (% dry matter) was measured at 450 °C and showed a close relationship to total carbon ($C = -0.664 + 0.527 \times LOI$, $r = 0.953$, $n = 39$, $P < 0.001$) and nitrogen ($N = -0.042 + 0.030 \times LOI$, $r = 0.942$, $n = 39$, $P < 0.001$). Iron and manganese were measured by inductively coupled plasma emission spectrometry after combustion and acid digestion. Inset: linear regression ($\pm 99\%$ confidence limits) of LOI and Fe/Mn ($r = 0.912$, $n = 59$, $P < 0.001$).

corresponding changes in species composition were much greater than in Portles See, where we found no differences between diatom assemblages as a result of temperature minima or acid depositions. The delay observed between Rassas and Portles may be an artefact resulting from the absolute dominance (40–80%) of a single species, *A. lirata*, in Rassas See between 1900 and 1950. But the generally lower pH of Portles See (~0.2 units lower than that of Rassas See) suggests that in lakes at very high altitudes (~2,900 m) in-lake processes may be less important in mitigating acidic depositions. This view is supported by the low concentrations of carbon and nitrogen in the sediment of Portles See (2–4 times below Rassas See). Interestingly, there is still synchronism between temperature and pH peaks in both lakes, for example around 1950 (Fig. 1a).

An alternative explanation for our findings can be sought in vegetational changes in the drainage area. Long-term biogenic changes in lake alkalinity are caused by the growth of vegetation and the formation of soils after deglaciation^{18,19}. Recent acidification has often been attributed to changes in land use, especially afforestation^{20,21}, whereas new acidification studies in Galloway (Scotland) and Norway have demonstrated that freshwater acidification was predominantly caused by acid precipitation^{17,22}. In high-alpine areas, the effect of soil and vegetation is expected to be less important in the process of biogenic acidification because only a small fraction of the drainage area is covered by soil⁵. The pollen diagram showed higher values for *Alnus viridis* and *Picea* in the older section (10–30 cm) of Rassas See, whereas in the younger section non-arboreal pollen, mainly grass pollen, dominated, reflecting the growing influence of meadows (Fig. 1a). As there was no significant rise in pollen concentration, we assume that, as should be expected for altitudes above 2,400 m a.s.l.²³, the watershed of Rassas See was never forested. The watershed of Portles See (2,892 m a.s.l.) has even less vegetation. Both lakes, however, showed a synchronous pH development during the nineteenth century, thus supporting the hypothesis that climate is the driving force for lake-water pH shifts.

The reversibility of acidification²⁴ depends on the balance of atmospheric components. The shift from sulphuric to nitric acids as the main ingredients of acid rain is clearly detectable in the Alps²⁵ and the question of whether greenhouse warming may counteract the effect of acid deposition is unsolved. In the near future, consequences and interactions of climate changes and anthropogenic impacts on the biogeochemistry of aquatic and terrestrial ecosystems should be studied by means of long cores

in remote alpine lakes situated above and below the present timber line. □

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Water mass exchange between the North Atlantic and the Norwegian Sea during the past 28,000 years

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THE Greenland, Iceland and Norwegian (GIN) seas are important regulators of heat transport in the Northern Hemisphere and of ocean–atmosphere CO₂ exchange^{1–5}. Rapid changes in the circulation of surface and deep waters in this region may induce nonlinear climatic effects and climate instabilities^{2,3}. Here we present carbon and oxygen isotope data that provide a record of circulation changes in the GIN seas during and at the termination of the Last Glacial Maximum (LGM). Inflow of nutrient-depleted waters from the GIN seas to form intermediate waters of the North Atlantic resulted in nutrient enrichment of North Atlantic deep water and consequent enhanced drawdown of atmospheric CO₂, contributing to the lower atmospheric pCO₂ during the LGM. The onset of deglaciation occurred at a time of low salinity in the GIN seas and thus of reduced thermohaline circulation. Although strong thermohaline circulation was later reinitiated in the North Atlantic as deglaciation proceeded, it cannot therefore have caused the onset of warming. Similarly, we find that rapid changes in thermohaline circulation cannot account for the transient return to a cooler climate during the Younger Dryas episode.

The advection towards the north of warm, saline surface water in the North Atlantic and GIN seas transfers large amounts of heat to the atmosphere on cooling and leads to deep convection and thermohaline circulation. These processes are fundamental for forming nutrient-depleted North Atlantic Deep Water (NADW), which is essential for the ocean–atmosphere CO₂ balance. NADW is also a principal contributor of heat to the Southern Ocean. Changes in this circulation will consequently affect key elements of the global climate system. Model experiments have indicated that the thermohaline circulation and the climatic state may switch between different modes and that the system is highly sensitive^{6,7}. Rapid switching of circulation in the North Atlantic and GIN seas is postulated to be the fundamental cause or a strong amplifier of high-amplitude climate

fluctuations³, and a potential mechanism for future strong climate feedbacks.

To improve the understanding and description of rapid changes in the ocean circulation of this region, we studied Norwegian Sea core HM52-43 (Fig. 1, Table 1). From isotope and faunal gradients between this core and North Atlantic core V23-81, we infer how surface and deep-sea parameters varied across the main barrier between the Norwegian Sea and the North Atlantic, the Faeroe–Shetland ridge. Core V23-81 is a key North Atlantic core for which detailed palaeoclimate records exist^{8–10}. Both cores are dated by accelerator mass spectrometry (AMS) and contain the Vedde ash-layer which directly ties the peak Younger Dryas horizon^{11,12}.

The benthic $\delta^{18}\text{O}$ results for core HM52-43 (Fig. 1a) suggest that the deep waters in the GIN seas at the LGM partly formed by freezing processes, and that the ratio of deep water produced by sea-ice formation to that produced by open-ocean overturn was higher during the LGM than today. We deduce this from

TABLE 1 Age model for core HM52-43

Depth (cm)	Age (yr)	Corrected age (yr)
2	2,000 ± 180	1,600
6	2,030 ± 150	1,630*
10	2,840 ± 170	2,440*
22	5,820 ± 180	5,420*
30	7,110 ± 230	6,710*
38	8,450 ± 160	8,050*
42	9,680 ± 150	9,280*
48	10,680 ± 170	10,280
48	Vedde ash ± 60	10,600*
52	10,490 ± 240	10,090
56	11,880 ± 220	11,480*
60	12,320 ± 190	11,920*
76	14,410 ± 290	14,010*
109	Stage 3.1 ± 3,000	25,400*

AMS ages from core HM52-43 are corrected for the reservoir effect (400 years). Age model based on ages indicated by asterisks.

the low glacial/interglacial $\delta^{18}\text{O}$ amplitude in core HM52-43 (Fig. 1a), which is –0.35‰ lower than the global ice volume effect of 1.2‰, and propose that the low amplitude arose because deep waters formed by brines had a stronger relative influence at the LGM than at present. This process produces dense water by salt expulsion during sea-ice formation but retains the low $\delta^{18}\text{O}$ of the less saline waters. It must overcome a general surface salinity lowering in the GIN seas of ~0.5‰ at the LGM¹³ and will produce deep waters with a distinctly negative $\delta^{18}\text{O}$ -signal, similar to that of present Antarctic Bottom Water^{14,15}. Such a process will more easily affect deep-water $\delta^{18}\text{O}$ in a restricted

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basin, such as the GIN seas or Arctic Ocean, than in the world ocean.

This record is a rare epibenthic foraminifer (*Cibicides wuellerstorfi*) record from the Norwegian Sea at the LGM. Earlier studies concluded that the low benthic glacial-interglacial amplitude of $\delta^{18}\text{O}$ was due to reworking, and failed to produce a reliable epibenthic isotope record for the LGM in the GIN seas¹⁶. We believe instead that the low amplitude in core HM52-43 results from changes in isotopic composition of the source water. We conclude from the arguments that follow that it is an *in situ* record: the same low amplitude is found for different benthic species (Fig. 1a); the benthic and planktonic records are very similar (this is unlikely if reworking was involved); and records from completely different settings in different portions of the GIN seas document the same low amplitude (ref. 17, and E.J. and I.B., unpublished data). Hence we conclude that the low amplitude indicates that northern source water formed at the LGM differed markedly in its isotopic composition and mode of formation from the water formed today.

From $\delta^{13}\text{C}$ in epibenthic foraminifers whose shells are relatively reliable recorders of the $\delta^{13}\text{C}$ of ambient water, we may reconstruct the ventilation of these waters. $\delta^{13}\text{C}$ is correlated with the oxygen content of ocean water and is inversely correlated with the oceanic nutrient content¹⁸. High epibenthic $\delta^{13}\text{C}$ at LGM (Fig. 1b) therefore documents strong ventilation in the deep GIN seas, very different from similar depths in the North Atlantic (core V23-81), which was under the influence of nutrient-enriched, low- $\delta^{13}\text{C}$ southern source waters¹⁰ (Fig. 1b).

The 30% decrease in atmospheric p_{CO_2} at the LGM, documented by ice cores, was most probably caused by enhanced productivity and/or circulation changes in the ocean. Boyle¹ pointed out that the existence of nutrient-depleted intermediate waters at the LGM and a redistribution of nutrients from intermediate to deep water provide a drawdown mechanism for atmospheric CO_2 . The source and origin of nutrient-depleted intermediate waters in the Atlantic is, however, much discussed: sources in the Mediterranean and circum-Antarctic, and unspecified sources in the North Atlantic, have been proposed¹⁹⁻²¹. Our results point to the highly ventilated deep waters in the GIN seas at the LGM as a probable source for a significant portion of the nutrient-depleted glacial intermediate water. The changed mode of deep-water formation documented by $\delta^{18}\text{O}$ probably produced deep waters with lower relative density than at present. Outflow from the GIN seas therefore did not contribute to form deep NADW as it does today, but apparently formed intermediate waters instead. An inflow, advecting near-surface waters northwards, is needed to compensate such an outflow, leading to some meridional circulation in the North Atlantic also during the LGM. Time-slice reconstructions of planktonic $\delta^{18}\text{O}$ at the LGM^{13,22,23} strongly indicate that this actually took place in the northeast Atlantic.

These results point to convection and brine formation at high northern latitudes in the Atlantic as an important facet of the LGM circulation. Overturning to intermediate depths at high latitudes and a more rapid spin of the circulation at intermediate depths would tend to deplete the upper ocean of nutrients, ventilate the thermocline, and increase nutrients and alkalinity in the deep reservoir instead. Boyle^{1,24,25} proposed that such a mechanism was a main cause of the 30% reduction in CO_2 in the glacial atmosphere. The alkalinity effect is a slow response because of the long time constant of the ocean carbonate system. A possible swifter mechanism may arise from increased ventilation of thermocline waters. Box modelling^{1,25} suggests that the direct effect on atmospheric CO_2 concentrations by intermediate water formation at high latitudes is smaller than those of other mechanisms, although these may have been initiated by altered North Atlantic circulation. This indicates that the main result of the Atlantic circulation changes at the LGM may have been to contribute to increased oceanic capability for CO_2 uptake in other regions of the world ocean.

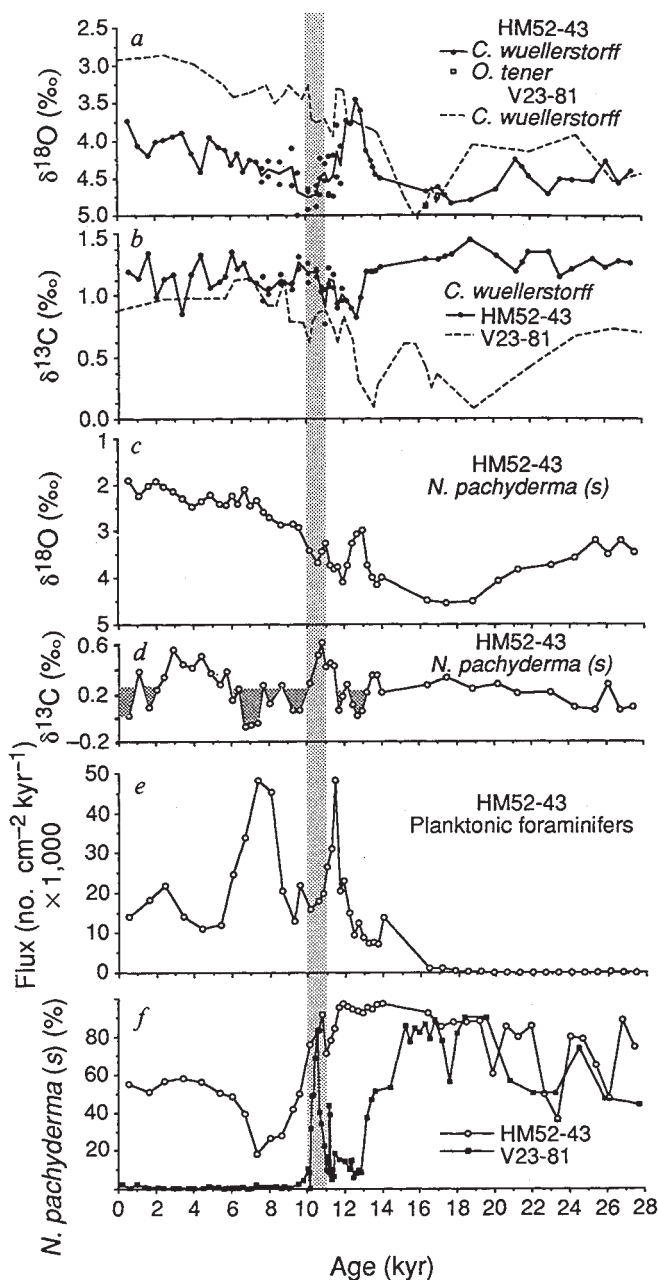


FIG. 1 Comparison of (a) $\delta^{18}\text{O}$ and (b) $\delta^{13}\text{C}$ of benthic foraminifers (HM52-43 from $64^\circ 31' \text{N}$, $00^\circ 44' \text{E}$, depth 2,781 m; V23-81 from $54^\circ 15' \text{N}$, $16^\circ 50' \text{W}$, depth 2,393 m). V23-81 data are from ref. 10. c, $\delta^{18}\text{O}$ and d, $\delta^{13}\text{C}$ from analyses of the planktonic foraminifer *Neogloboquadrina pachyderma* (sinistral form, s) from HM52-43. e, Total flux of planktonic foraminifers from core HM52-43, calculated according to ref. 35 by using bulk density and porosity data from the nearby core HM79-20 ($64^\circ 40' \text{N}$, $00^\circ 42' \text{W}$). f, Relative occurrence of *N. pachyderma* (s) in % (HM52-43 from this work and V23-81 from ref. 8). The $\delta^{18}\text{O}$ -values from *C. wuellerstorfi* and *Oridorsalis tener* are corrected by $+0.64\%$ and $+0.36\%$. The vertical shaded area marks the Younger Dryas chronozone $\delta^{18}\text{O} = [({}^{18}\text{O}/{}^{16}\text{O}_{\text{sample}})/({}^{18}\text{O}/{}^{16}\text{O}_{\text{standard}})] - 1$, where the standard is PDB. $\delta^{13}\text{C}$ is analogous.

The strong benthic $\delta^{13}\text{C}$ gradient between the Norwegian Sea and the northeast Atlantic before 12.5 kyr BP (Fig. 1b) shows that nutrient-poor oxygenated deep-water from the GIN seas did not ventilate the deep regions of the northeast Atlantic until 12.6 kyr BP. This marks the onset of thermohaline circulation of the present mode. The start-up time is documented by increased $\delta^{13}\text{C}$ along the entire Atlantic²⁶. Preceding this switch, we observe a strong depletion of surface salinity by deglacial melt water, demonstrated by a planktonic $\delta^{18}\text{O}$ peak of 1‰ (Fig. 1c) at 13.0 kyr BP. The peak corresponds to a drop in near-surface salinity of 1–1.5‰. The accompanying $\delta^{13}\text{C}$ drop indicates a reduction of the ventilation of near-surface waters, probably as a result of a stability cap. High-resolution cores from the GIN seas record one or two large meltwater spikes at 14.0 to 12.5 kyr BP^{23,27,28}. This was apparently the period of most active meltwater transfer from the destruction of marine-based ice sheets in Fennoscandia, Greenland and the Barents, Kara and East Siberian Seas²⁹, in addition to meltwater advected from the Laurentide ice sheet. The meltwater spikes pre-date the peak eustatic sea-level rise³⁰ by 1,600 to 700 ^{14}C years, indicating that the main meltwater dilution in the studied area was a precursor to the deglaciation of the main ice mass of the Laurentide ice sheet (also suggested by refs 27–29). This points to a time lag between the oxygen isotope record and the sea-level record, caused by deglaciation of marine-based parts of ice sheets. The initial deglacial sea-level rise was probably smaller than indicated from ice-volume calculations based on isotopes, because submarine ice was replaced by water.

The major deglacial light $\delta^{18}\text{O}$ peak at ~13.0 kyr BP affected both the deep Norwegian Sea and the deep North Atlantic (Fig. 1a). We observe no time lag between the surface (planktonic) and the deep-water (benthic) response. This indicates that the transfer of the deglaciation signal to the deep ocean is rapid. The lowered $\delta^{13}\text{C}$ indicates that open-ocean convection and deep-sea oxygenation were reduced at this time. For there to be such an instant transfer of the $\delta^{18}\text{O}$ spike to large depths despite the reduced ventilation, we propose that the meltwater signal was transferred by the brine mechanism. The slight time difference between the low $\delta^{13}\text{C}$ event in the Norwegian Sea and the same event in the North Atlantic at ~13.0 kyr BP (Fig. 1f) is within the error of the timescales of both cores. The planktonic and benthic evidence for major meltwater transfer and concurrent low $\delta^{13}\text{C}$ strongly suggests that the main period of reduced convection occurred at a time of maximum low-salinity forcing from the first phase of deglaciation. This terminated at 12.6 kyr BP, and thermohaline circulation rapidly increased (Fig. 1b, and refs 26, 31). The rapid transfer of the deglacial $\delta^{18}\text{O}$ signal to large depths shows, however, that deep-water formation must have continued, probably at a lower rate and with changing sources.

Figure 1f shows that warming of the surface North Atlantic was under way at 14–13.0 kyr BP. We observe this warming at the same time in the Norwegian Sea by a sharp increase in foraminifer flux (Fig. 1e) which reflects a change to prolonged sea-ice free conditions (T. Johannessen, unpublished data). Very-high-resolution records from the eastern Norwegian Sea also document strong warming at 13.4 kyr BP (refs 32, 33). This implies that warming, and subsequent melting and deglacial meltwater transfer, was initiated during times of reduced thermohaline circulation. Benthic evidence from the whole Atlantic sector points to 12.5 kyr BP as the time when strong NADW formation was reinitiated^{10,26,31}; this is almost 1,000 ^{14}C years later, at a time when the meltwater anomalies vanished in the Norwegian Sea (Fig. 1a, c and refs 23, 27, 28). Whereas the major melting of the Northern Hemisphere ice masses happened after NADW formation was reinitiated at 12.6 kyr BP²⁶, and therefore may have been caused largely by heat release from the 'conveyor circulation', the onset of warmth in northern Europe and the onset of melting clearly happened before this. It is therefore difficult to see how the onset of warming could be caused by the switching on of the thermohaline cell and increased thermohaline overturn in the North Atlantic region, as proposed in refs 3 and 34. Warming apparently took place at times of both strong and weak thermohaline circulation.

The rapid coolings that punctuated deglaciation, of which the Younger Dryas was the most prominent, are also not easily explained in the context of thermohaline switches such as those proposed by refs 3, 34 and 35. The heavy benthic $\delta^{18}\text{O}$ values at the Younger Dryas level in HM52-43 (Fig. 1a) record open-ocean convection in the Younger Dryas. The values are comparable with those of the LGM. Taking the ice volume of the Younger Dryas into account, such high values can only be possible if the mode of formation and temperature of the deep water of the GIN seas were similar to those of the present ocean. This shows that the modern type of deep-water formation operated in the Younger Dryas, in contrast to the situation at the LGM and during early deglaciation at 13.5–13.0 kyr BP. High benthic $\delta^{13}\text{C}$ shows that there was good ventilation in the Younger Dryas and further supports this conclusion. The high foraminifer flux in the Younger Dryas (Fig. 1e) is another strong indication of long ice-free seasons and a clear indication that both surface and deep-water conditions were very different in the Younger Dryas than at the LGM, which had low flux. Although the Younger Dryas was a return to glacial climates in northern Europe, and to glacial sea surface temperatures, it was clearly not, as proposed in ref. 3, a return to glacial ocean circulation. A return to the glacial mode of circulation therefore cannot be a viable explanation of the cooling. □

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Seawater Sr isotope variation over the past 300 kyr and influence of global climate cycles

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THE past 300,000 years have been characterized by glacial/interglacial fluctuations accompanied by glacio-eustatic changes in sea level and changes in continental erosion arising from varying low-latitude rainfall and river drainage. Here we use measurements of strontium isotopes in foraminifera and corals to place limits on variations in the Sr isotope composition of sea water in response to these changing inputs. We find small variations of about 20 p.p.m. in the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio, which for the foraminiferal record seem to follow a cycle close to the 100-kyr periodicity seen for a number of other climate-related phenomena. These are superimposed on a general increase in $^{87}\text{Sr}/^{86}\text{Sr}$ through the Cenozoic to the present. On short timescales these changes are likely to be controlled by variations in the global riverine Sr flux, and thus by weathering rates, rather than in the hydrothermal Sr flux at mid-ocean ridges. We show that transient variations over a 50-kyr timescale can be explained on the basis of the present-day global drainage system in conjunction with the perturbations expected to accompany climate changes over the past 300 kyr.

As the precision of Sr-isotope analysis has improved, it has been possible to determine variations in the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of sea water over a large part of ocean history. Particularly detailed records are now available for the past few tens of millions of years¹⁻⁸, and these have shown a general increase in seawater $^{87}\text{Sr}/^{86}\text{Sr}$ ratio towards the present day, but with some finer structure evident on timescales of 0.5–1 Myr or even less^{7,9}. We have used two types of marine carbonate sample to investigate the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of sea water over the past 300 kyr, namely foraminifera and corals. Planktonic foraminifera *Pulleniatina obliquiloculata* are from a single equatorial Pacific site and were selected from piston core Vema 28-238, for which ages have been derived from several studies^{10,11}. The smooth surface of this particular species allows adhering clay to be cleaned off easily. The corals are from two uplifted terrace sequences, one in New Guinea and the other in Barbados¹²⁻¹⁴, for which we have determined new ²³⁰Th ages. Coral aragonite was selected from sections several millimetres thick, after X-ray, electron microscopy and thin-section petrographic analysis, to avoid any calcite present (this was not greater than 1% in any of the samples analysed). These and the foraminifera were ultrasonically cleaned before analysis.

Thorium-230 ages have been determined by thermal ionization mass spectrometry for 12 samples of coral from the Huon Peninsula, New Guinea, and for three samples from Clermont Nose, Barbados (Table 1) closely following techniques described previously^{15,16}. The ages are determined to a precision of ± 0.5 kyr (2σ) for the youngest samples dated at 41.1 kyr, to ± 1 kyr (2σ) for those at 120 kyr, and ± 2 –4 kyr (2σ) for those at 240 kyr. The initial $^{234}\text{U}/^{238}\text{U}$ ratios ($\sigma^{234}\text{U}_{\text{init}}$, Table 1), for corals up to ~120 kyr in age, are close to that of modern sea water ($\delta^{234}\text{U} = 135$ –155; ref. 15). The $\delta^{234}\text{U}_{\text{init}}$ of corals of age 240 kyr is higher ($\delta^{234}\text{U}_{\text{init}} \approx 200$), whereas the single sample KAU-A-1 of age 198 kyr has a very low $\delta^{234}\text{U}_{\text{init}}$ of ~65. It is unclear whether these variations arise from real differences in

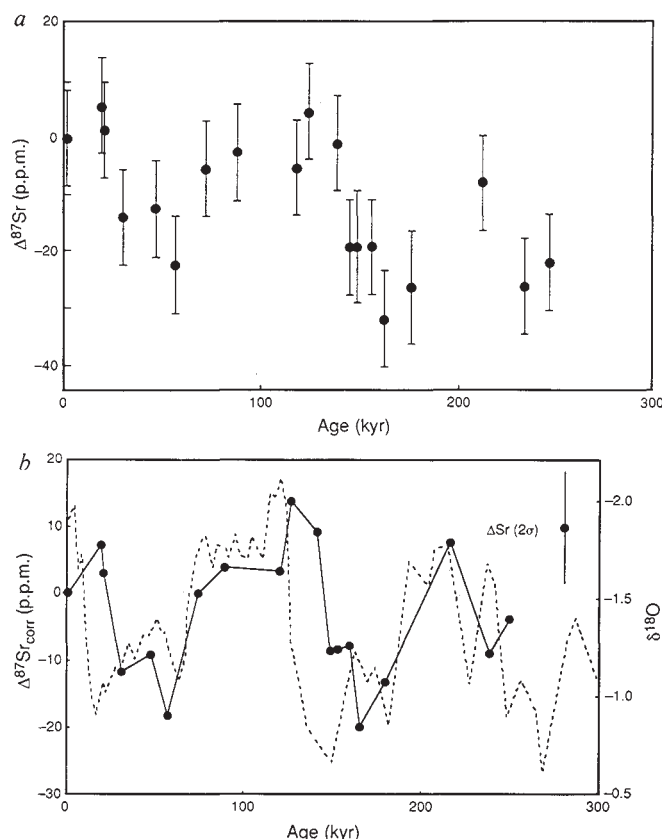


FIG. 1 a, Strontium isotope composition of foraminifera, from core Vema 28-238, expressed as $\Delta^{87}\text{Sr}$ (see Table 2; values in p.p.m.). b, Strontium isotope data for foraminifera from core Vema 28-238 expressed as $(\Delta^{87}\text{Sr})_{\text{corr}}$ after removal of the long-term trend identified by Hodel et al.⁷ The adjustment is made according to $(\Delta^{87}\text{Sr})_{\text{corr}} = \Delta^{87}\text{Sr} - [\text{Age (Myr)} / (1.32 \times 10^{-2})]$. Comparison with the $\delta^{18}\text{O}$ record for the same core^{10,11} (dashed line) suggests that $\Delta^{87}\text{Sr}$ becomes more positive with the onset of glacial conditions, consistent with the long-term trend of increasing $\Delta^{87}\text{Sr}$ in parallel with Cenozoic cooling.

the $^{234}\text{U}/^{238}\text{U}$ ratio of sea water or from the effects of diagenesis¹⁷, which might also affect $^{87}\text{Sr}/^{86}\text{Sr}$ ratios.

We have measured $^{87}\text{Sr}/^{86}\text{Sr}$ ratios for the 15 coral and 19 foraminifera samples (Table 1) to very high precision (~ 10 p.p.m., $2\sigma_m$). Details of mass spectrometric procedures and the standardization justifying this quoted precision are given in Table 1. The $^{87}\text{Sr}/^{86}\text{Sr}$ ratios for corals (Table 1) show a very small range from 0.709157(6), where the error in the final digit ($2\sigma_m$) is given in parentheses, to 0.709176(6), without any clear dependence on ²³⁰Th age (Table 1). The $^{87}\text{Sr}/^{86}\text{Sr}$ ratios for the foraminifera have a similar range to that of the corals, from 0.709145(6) to 0.709172(6), which exceeds that expected from the assigned errors. It is noteworthy that the $^{87}\text{Sr}/^{86}\text{Sr}$ ratios for most corals and foraminifera are lower than that of modern sea water deduced from zero-age foraminifera in V28-238, and from present-day shell material from the Bahamas and South Africa, at 0.709165(6) and 0.709173(6), respectively (Table 2). The $^{87}\text{Sr}/^{86}\text{Sr}$ ratios are conveniently expressed as deviations in p.p.m. from a modern foraminifera as follows:

$$\Delta^{87}\text{Sr} \text{ (p.p.m.)} = \left(\frac{(^{87}\text{Sr}/^{86}\text{Sr})_{\text{sample}}}{0.709168} - 1 \right) \times 10^6$$

Inspection of our Sr isotope results for foraminifera alone (Fig. 1a) suggests that real structure is present on a timescale of 100 kyr or less, and that this is superimposed on the long-term trend of increasing $^{87}\text{Sr}/^{86}\text{Sr}$ over the past 3 Myr identified previously⁷. Figure 1b shows the foraminifera data after removal of the long-term trend described by $\Delta^{87}\text{Sr} = \text{age} \times 75.8$ in

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TABLE 1 Coral samples from New Guinea and Barbados

Sample	Location	$\delta^{234}\text{U}_{\text{present}}$ (‰)*	$\delta^{234}\text{U}_{\text{init}}$ (‰)†	$(^{230}\text{Th}/^{238}\text{U})\ddagger$	U (p.p.m.)	Th (p.p.b.)	Th age (kyr)	$^{87}\text{Sr}/^{86}\text{Sr}\S$
New Guinea								
KWA-I-1	Huon Peninsula	120.9±3.1	135.9±3.5	0.3551±0.0035	2.901±0.003	0.4487±0.0044	41.1±0.5	0.709157±6
KWA-N-1	Huon Peninsula	124.0±2.7	144.0±3.1	0.4361±0.0027	2.683±0.003	0.1481±0.0009	52.8±0.5	0.709172±7
KAN-D-5	Huon Peninsula	120.6±2.5	137.6±2.9	0.3916±0.0047	3.945±0.003	0.1476±0.0018	46.4±0.7	0.709166±6
KWA-K-1	Huon Peninsula	118.6±3.1	137.8±3.6	0.4358±0.0026	3.181±0.002	0.1314±0.0008	53.2±0.5	0.709169±6
FRT-F-1	Huon Peninsula	113.2±2.8	137.7±3.4	0.5286±0.0038	2.722±0.002	0.5882±0.0043	69.1±0.7	0.709169±6
BWW-D-2	Huon Peninsula	120.8±3.3	167.5±4.6	0.7437±0.0039	2.968±0.001	0.1657±0.0009	115.1±1.2	0.709155±6
KAU-A-1	Huon Peninsula	37.1±4.5	65.0±7.9	0.8766±0.0021	3.468±0.001	0.1861±0.0004	198.1±3.3	0.709161±6
KAU-B-1	Huon Peninsula	138.2±2.6	280.0±5.2	1.0576±0.0045	2.451±0.001	0.6315±0.0005	249.1±4.6	0.709157±6
SIAL-B-1	Huon Peninsula	111.7±2.8	232.3±3.9	1.0373±0.0022	2.538±0.001	0.1968±0.0004	258.4±3.8	0.709158±6
Barbados								
41 a	Clermont Nose traverse (Worthing Terrace)	131.6±3.2	169.5±4.1	0.6417±0.0052	2.591±0.001	0.0995±0.0008	89.1±1.2	0.709164±6
51 a	Clermont Nose traverse (Rendezvous Terrace)	102.6±3.9	144.3±5.5	0.7482±0.0037	3.579±0.004	0.2466±0.0012	120.2±1.4	0.709176±6
53 a	Clermont Nose traverse (Thorpe Terrace)	113.8±3.4	226.3±6.8	1.0217±0.0044	2.968±0.002	0.1560±0.0007	242.4±4.9	0.709161±6

Uranium isotope ratios ($^{230}\text{Th}/^{238}\text{U}$) activity ratios, U and Th contents, ^{230}Th ages and $^{87}\text{Sr}/^{86}\text{Sr}$ isotope ratios from coral samples from New Guinea and Barbados. Errors are $\pm 2\sigma$.

* $\delta^{234}\text{U}_{\text{present}} = [(^{234}\text{U}/^{238}\text{U})_{\text{sample}} / (^{234}\text{U}/^{238}\text{U})_{\text{eq}}] - 1$, where $(^{234}\text{U}/^{238}\text{U})_{\text{eq}}$ is the ratio at secular equilibrium.

† $\delta^{234}\text{U}_{\text{init}} = \delta^{234}\text{U}_{\text{present}} \times e^{\lambda^{234}T}$, where λ^{234} is the decay constant of ^{234}U and T is the age of the sample.

‡ $(^{230}\text{Th}/^{238}\text{U})$ is the activity ratio.

§ Samples analysed for $^{87}\text{Sr}/^{86}\text{Sr}$ by thermal ionization mass spectrometry with a VG 354 mass spectrometer using dynamic multiple collection and a power-law mass fractionation correction. Corrections for ^{87}Rb , abundance sensitivity or the time constants of the $10^{10}\text{-}\Omega$ resistors were unnecessary. The $^{87}\text{Sr}/^{86}\text{Sr}$ ratio measurements were made between July 1990 and February 1991. Forty-eight analyses of the Sr standard (NBS-987) were made over this interval, to bracket every 2–3 samples, and gave average values of 0.710244 ($n=10$), 0.710246 ($n=5$), 0.710243 ($n=14$), 0.710244 ($n=11$) and 0.710243 ($n=8$), respectively, for July 1990, September 1990, October 1990, December 1990 and February 1991. Samples and standards were measured to a precision of 5–6 p.p.m. ($1\sigma_m$) and the external precision of all the 48 NBS-987 analyses is 6.8 p.p.m. (1σ), demonstrating that the internal statistics reported for these measurements are good estimates of long-term reproducibility. Statistics on the standards are even better over shorter intervals of time but no attempt has been made to adjust data in any way and all analyses are reported as measured. Results are normalized to $^{86}\text{Sr}/^{88}\text{Sr}=0.1194$.

comparison with the $\delta^{18}\text{O}$ variation in V28-238 (refs 10, 11). The r.m.s. deviation of $\Delta^{87}\text{Sr}$ for all 19 foraminifera from the linear change as defined in ref. 7 is a factor of two greater than the average σ_m for $\Delta^{87}\text{Sr}$ measurements, whereas it should be equal for a perfect fit. The reproducibility of standard measurements (Table 1) suggests that variations in $^{87}\text{Sr}/^{86}\text{Sr}$ ratio at the 20 p.p.m. level should be readily resolvable. In addition, for a smooth variation in $^{87}\text{Sr}/^{86}\text{Sr}$ ratio with time the mean difference between adjacent foraminifera samples in the core divided by $\sqrt{2}$ should be similar to the quoted measurement precision. For the data in Table 2, this value is 5.7 p.p.m., which compares favourably with the quoted measurement precisions of 5–6 p.p.m. ($1\sigma_m$). Taken together, the repeatability of standards and internal downcore consistency of measured $^{87}\text{Sr}/^{86}\text{Sr}$ ratios

suggests that these short-term variations are indeed real. Although there are too few data to make a meaningful spectral analysis at this stage, the data do strongly suggest that a cyclical variation exists in $^{87}\text{Sr}/^{86}\text{Sr}$ with a period of 100 kyr. Such a period is well known in other climate-related phenomena through the Pleistocene, such as oxygen-isotope variations in this and many other cores^{11,18}. The preliminary conclusion to be drawn is that the Sr-isotope composition of Pacific sea water does seem to have followed other orbital driven phenomena evident in climate change. Such variations are less clear in the coral $^{87}\text{Sr}/^{86}\text{Sr}$ data. The main discrepancy between the coral and the foraminifera data is in $^{87}\text{Sr}/^{86}\text{Sr}$ for the two ~50-kyr samples from New Guinea. The reason for this is not known, but the possibility of diagenetic modification of $^{87}\text{Sr}/^{86}\text{Sr}$ in the

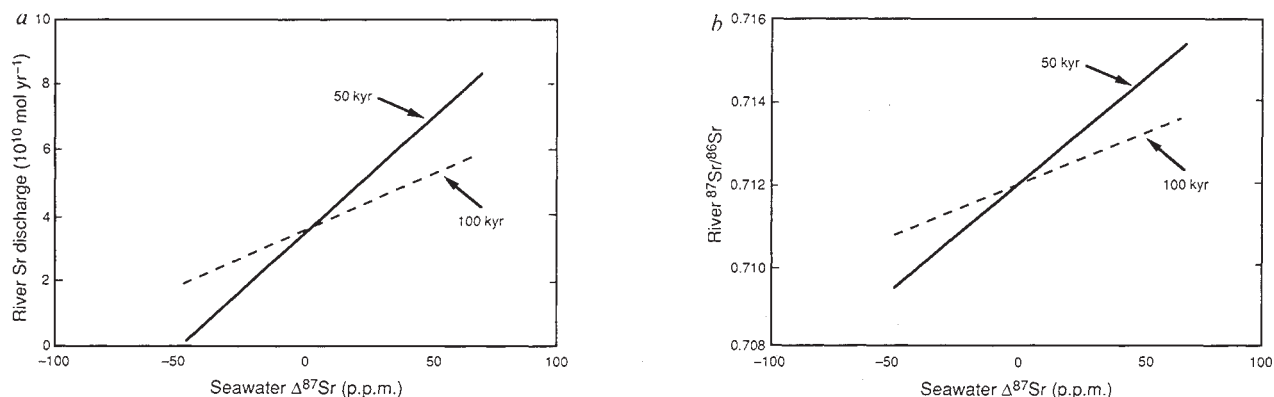


FIG. 2 *a*, Response of the Sr isotope composition of sea water expressed as $\Delta^{87}\text{Sr}$ (see Fig. 1*b*) on the riverine Sr flux. The $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of the riverine flux is fixed at 0.7119 (ref. 20), which corresponds to $\Delta^{87}\text{Sr}=+3,850$ p.p.m. The dependence of $\Delta^{87}\text{Sr}$ on riverine flux is shown for transient change occurring over 50 and 100 kyr. The total Sr content of the oceans is taken as 1.25×10^{17} mol, and the total present-day Sr input at 5.1×10^{10} mol Sr yr^{-1} . These relationships are calculated using equations

for Sr mass balance and residence time from appendix I in ref. 7. *b*, Response of the Sr isotope composition of sea water to the mean $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of river water at a fixed flux equal to the present-day value of 3.33×10^{10} mol yr^{-1} . The present-day weighted mean $^{87}\text{Sr}/^{86}\text{Sr}$ ratio is taken as 0.7119 (ref. 19), and the effect on $\Delta^{87}\text{Sr}$ of variations in this value is shown for both 50-kyr and 100-kyr transients. The equations of Hodell *et al.*⁷ are used for these calculations.

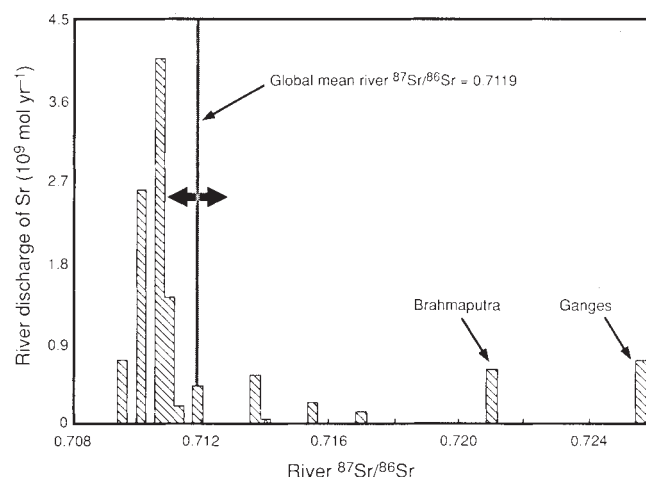


FIG. 3 Distribution of riverine $^{87}\text{Sr}/^{86}\text{Sr}$ in terms of discharge rate, for the world's main rivers, using an interval of 3×10^{-4} in river $^{87}\text{Sr}/^{86}\text{Sr}$. The data are from the compilation of Palmer and Edmond¹⁹. Note the relatively high $^{87}\text{Sr}/^{86}\text{Sr}$ ratios for the Ganges and Brahmaputra. The global mean riverine $^{87}\text{Sr}/^{86}\text{Sr}$ ratio (0.7119) is shown by the vertical line. The heavy arrows show the range by which the mean would have to change if the 50-kyr transient changes shown in Fig. 1 are produced by a change in the global average riverine $^{87}\text{Sr}/^{86}\text{Sr}$.

uplifted coral terraces cannot be eliminated at present.

A principal conclusion is that the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of the equatorial Pacific ocean may vary by ~ 20 p.p.m. on the 50-kyr timescale. This timescale is long compared with the mixing time of the oceans (1–2 kyr), and the observed variations, assuming them to be of global significance, must reflect changes in the major mass fluxes that control the ocean Sr budget and/or their isotope compositions. Of the fluxes involved^{1,19}, the hydrothermal Sr flux at spreading ridges is unlikely to vary significantly on this timescale. The riverine Sr flux and calcite dissolution rate should vary in response to changing patterns of precipitation and the carbonate compensation depth, respectively. Estimates for the present carbonate dissolution flux for Sr (ref. 19) are small (3.4×10^9 mol yr^{-1}) compared with 33.9×10^9 mol yr^{-1} for the river flux. Although carbonate dissolution does vary cyclically during the Pleistocene, it involves very young (essentially contemporary) carbonate and should have a subordinate role. We investigate further only the possibility that the observed shifts in seawater Sr are produced through changes in the riverine Sr flux and Sr isotope composition.

Because seawater Sr does not have a steady-state isotope composition, we analyse its response to changes in the riverine flux for a transient shift following the discussion of Hodell *et al.*⁷. The relationship between changes to the riverine Sr flux and $\Delta^{87}\text{Sr}$ for 50-kyr and 100-kyr transients are shown in Fig. 2a. The effect on $\Delta^{87}\text{Sr}$ of a change in the total riverine flux at constant $^{87}\text{Sr}/^{86}\text{Sr}$ ratio from the present value of 3.33×10^{10} mol yr^{-1} is different for a 50-kyr and a 100-kyr transient. A change of $\pm 50\%$ in the flux is sufficient to produce a change of

TABLE 2 Foraminifera samples

Depth (cm)	Age (kyr)*	$^{87}\text{Sr}/^{86}\text{Sr}$ $\pm(2\sigma_m)^\dagger$	$\Delta^{87}\text{Sr}$ (p.p.m.) $\pm(2\sigma_m)$
Foraminifera			
0	0	0.709168 (6)	0 $\pm$ 8.5
42	19	0.709172 (6)	5.6 $\pm$ 8.5
45	20	0.709169 (6)	1.4 $\pm$ 8.5
62	30	0.709158 (6)	-14.1 $\pm$ 8.5
82	46	0.709159 (6)	-12.7 $\pm$ 8.5
101	56	0.709152 (6)	-22.6 $\pm$ 8.5
131	73	0.709164 (6)	-5.6 $\pm$ 8.5
151	84	0.709166 (6)	-2.8 $\pm$ 8.5
206	119	0.709164 (6)	-5.6 $\pm$ 8.5
215	125	0.709171 (6)	4.2 $\pm$ 8.5
245	139	0.709167 (6)	1.4 $\pm$ 8.5
261	147	0.709154 (6)	-19.7 $\pm$ 8.5
271	151	0.709154 (7)	-19.7 $\pm$ 8.5
282	158	0.709154 (6)	-19.7 $\pm$ 8.5
291	164	0.709145 (6)	-32.4 $\pm$ 8.5
322	179	0.709149 (7)	-26.8 $\pm$ 9.9
383	216	0.709162 (6)	-8.5 $\pm$ 8.5
410	238	0.709149 (6)	-26.8 $\pm$ 8.5
443	249	0.709152 (6)	-22.6 $\pm$ 8.5
Modern shells			
Bahamas	0	0.709165 (6)	-4.23 $\pm$ 8.5
South Africa	0	0.709173 (6)	7.05 $\pm$ 8.5

The depth, age, $^{87}\text{Sr}/^{86}\text{Sr}$ ratios and $\Delta^{87}\text{Sr}$ values for foraminifera samples from the core Vema V28-238, and for modern shells from the Bahamas and South Africa. $\Delta^{87}\text{Sr}$ values are calculated as follows:

$$\Delta^{87}\text{Sr} (\text{p.p.m.}) = \left(\frac{(^{87}\text{Sr}/^{86}\text{Sr})_{\text{sample}}}{0.709168} - 1 \right) \times 10^6$$

* Ages from ref. 11.

† $^{87}\text{Sr}/^{86}\text{Sr}$ ratio measurements as for Table 1.

± 20 p.p.m. in $\Delta^{87}\text{Sr}$ even for a 50-kyr transient change. The alternative situation of a fixed Sr flux with variable $^{87}\text{Sr}/^{86}\text{Sr}$ is shown in Fig. 2b. In this case a shift of the present-day weighted mean average $^{87}\text{Sr}/^{86}\text{Sr}$ ratio from 0.7119 (ref. 19) by ~ 0.0005 is sufficient to produce a transient change in $\Delta^{87}\text{Sr}$ of 20 p.p.m. in 50 kyr. Comparison with the Sr isotope composition of global river discharge (Fig. 3) compiled from data in ref. 19 shows that such a shift is still well within the range of the Sr isotope composition of modern rivers. The Sr discharge is currently dominated by low-latitude rivers with $^{87}\text{Sr}/^{86}\text{Sr}$ ratios around 0.710 as shown. The Ganges and Brahmaputra, however, have $^{87}\text{Sr}/^{86}\text{Sr}$ ratios of >0.72 , and these raise the global average to 0.7119 as shown. Perturbation of Himalayan drainage with these high $^{87}\text{Sr}/^{86}\text{Sr}$ ratios would alone be sufficient to produce the observed shift. This is not proposed as a unique interpretation of the observations on seawater Sr, but it does illustrate that the variations observed are feasible and to be expected within the framework of the modern system of global drainage. The amount of low-latitude monsoonal rainfall has varied with solar radiation and orbital factors^{20,21}, and this may also have been accompanied by changes in Sr isotope composition. □

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Discovery of the spawning area for Japanese eel

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At the beginning of this century, the Danish oceanographer Johannes Schmidt outlined the spawning area of Atlantic eels *Anguilla* spp. in the Sargasso Sea^{1,2}. But the spawning location of the Japanese eel *A. japonica* in the Pacific Ocean has eluded researchers for over 60 years. I report here the discovery of their spawning location. By measuring oceanographic conditions and collecting the transparent leaf-like eel larvae, termed leptocephali (see Fig. 1), we determined the Japanese eel spawning area to be in the North Equatorial Current west of the Mariana Islands, at a salinity front near 15° N, 140° E. This discovery shows that the key similarity between the spawning sites of Atlantic and Pacific eels is the placement of leptocephali into the major ocean currents that will return them to their juvenile rearing habitats.

After growing in freshwater habitats, adult eels migrate thousands of kilometres to breed in the ocean. This exceptional migratory pattern, termed diadromy³, plus the economic importance of eels has attracted much research effort towards locating their oceanic breeding site. Small leptocephali collected in the Atlantic Ocean^{1,2,4-10} have indicated that both the European, *A. anguilla*, and American eel, *A. rostrata* spawn in the Sargasso Sea. But before the 1991 expedition of the research vessel *Hakuho-Maru* of the Ocean Research Institute, University of Tokyo, the catch of leptocephali of the Japanese eel was too small in number and too large in size to determine the spawning site. A previous collection of 110 leptocephali¹¹⁻¹⁶, plus the information gained from four previous expeditions¹¹⁻¹⁴ since

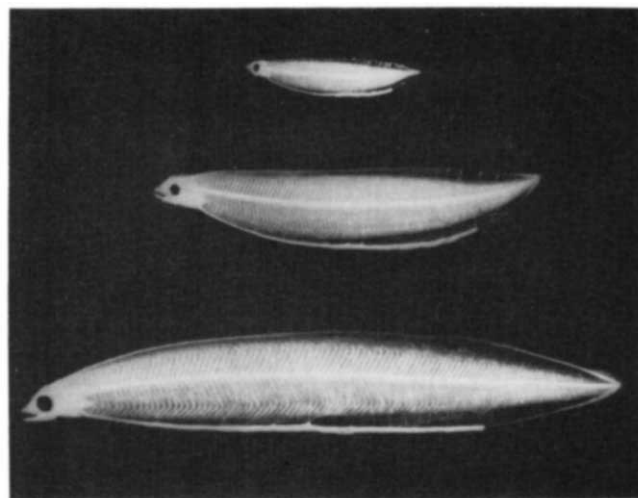
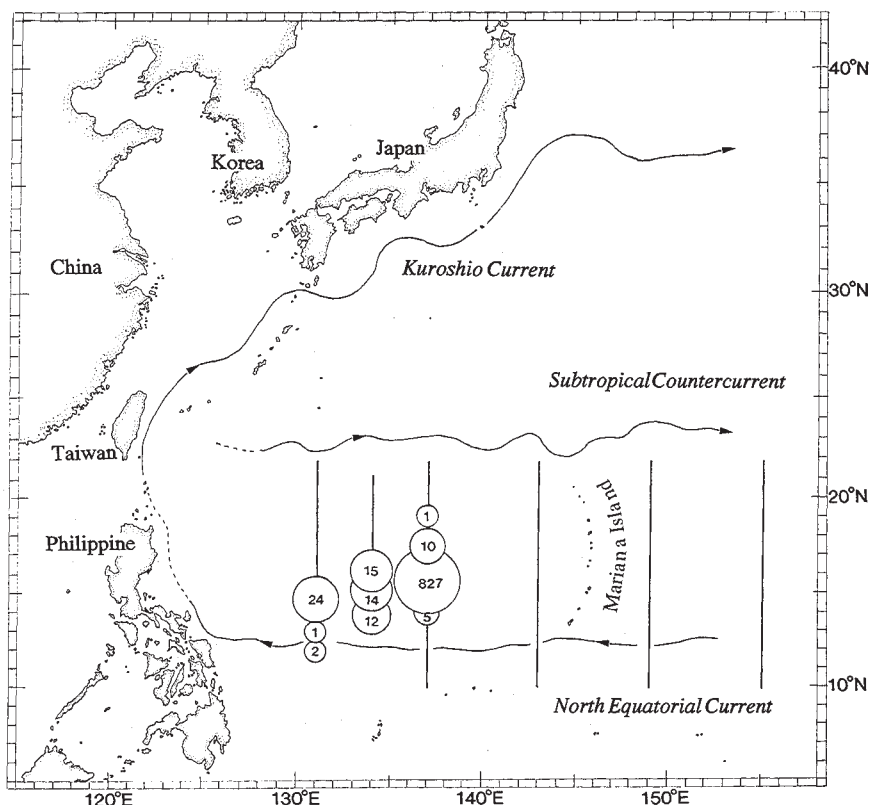


FIG. 1 Representative leptocephali of *Anguilla japonica* collected during the Hakuho-maru expedition in June–July 1991. Total lengths are 9.8, 21.6 and 33.5 mm.

1973, indicated that for the 1991 expedition we should establish a wide sampling grid between the Marshall and Philippine Islands.

We collected 911 leptocephali, located from 12–19° N and 131–137° E (Fig. 2). Most were within the margins of the North Equatorial Current, flowing westerly at about 12° N, whereas others were in complex eddies lying at the edge of this current but well south of the Subtropical Countercurrent flowing easterly at about 22° N. The leptocephali were found at 50–100 m depth with greatest abundance at 75 m. The leptocephali averaged

FIG. 2 The sampling stations at which Japanese eel leptocephali were found in June–July 1991. Six transects running north-south from 10–22° N, and between 131–155° E, were set as shown. A sampling station was located every 2° latitude on each transect. At each of these 34 main stations, both net sampling and conductivity, temperature, depth and dissolved oxygen (CTDO) measurements were taken about every 10 h around the clock. Net sampling involved a 60 min oblique tow using a 3 m Isaacs Kidd Midwater Trawl (IKMT) net with 8.7 m² mouth opening and 1-mm mesh, from the surface to a depth of 300 m. CTDO measurements involved a cast to a depth of 1,000 m. In addition to the main stations, night samples were taken at 47 sites between the main stations using 45-min oblique tows to a depth of 150 m. Even more intensive sampling was undertaken once the first leptocephalus was collected. At 56 sites near the stations bordering 15° N 131° E, 14–16° N 134° E and 14–17° N 137° E, additional net sampling was done. Horizontal step tows were made at night in three depth strata with a 0.5- or 1.0-mm mesh IKMT net. The depth strata were selected on the basis of the location of the greatest zooplankton biomass as observed using sonar. The towing depth was adjusted using a pressure-sensitive ultrasonic transmitter. Finally the vertical distribution of leptocephali was determined at two stations using 10 Motoda-type multiple layer closing nets of 80-cm diameter and 0.5-mm mesh operated from the surface to a depth of 500 m. Leptocephali were identified by counting myomeres²⁹ and measured for total length. The specimens collected within 1° of latitude were pooled for the sample sizes shown in circles on the map. The current locations are based on a Doppler



current profiler. Average current speed was measured from the trajectory of a satellite tracking buoy, named Argos Buoy.

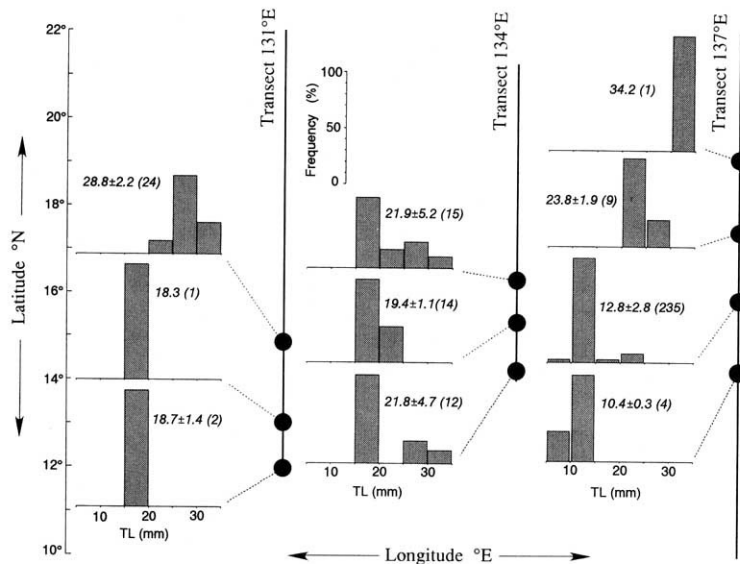


FIG. 3 The size frequency distribution and mean total length (TL $\pm$ s.d., in mm) of leptocephali collected at the 10 successful stations on three transects ($n = 317$). Individual sample sizes are given in parentheses.

15.5 mm $\pm$ 3.5 s.d. long and ranged from 7.9 to 34.2 mm (Fig. 3). Smaller and thus younger leptocephali tended to be at the more southern and eastern stations.

At hatch, about 36 h after fertilization, eels are 3 mm long^{17,18}. Because leptocephali grow at 0.56 mm per day (refs 19, 20), the smallest leptocephali at the two most south-eastern locations (average length 12.8 $\pm$ 2.8 mm s.d., range, 7.9–24.5 mm, $n = 239$) would be about 19 days old after fertilization (range, 10–40 days). The westerly current in which these leptocephali were found was flowing at about 20 cm s⁻¹ (17.3 km per day). Therefore, these leptocephali probably travelled 328 km (range, 173–691 km) from the spawning location of their parents. This identifies the geographic location of the spawning adults at roughly 15° N, 140° E (range, 139–143° E). To test this calculation, the estimated spawning location for leptocephali collected 131° E (average length, 27.6 mm) would be about 138° E. This does not differ greatly from the above estimate of 140° E.

This spawning location corresponds to a salinity front, separating the less saline water mass of the North Equatorial Current from the typically high salinity of tropical water (Fig. 4). The reduced salinity in the frontal zone, or some associated features such as odour²⁰, may provide eel adults approaching from a northerly direction near the surface^{22,23} with a cue which triggers cessation of migration and initiation of spawning²¹. The reproductive season of the eels is lengthy, from April to Novem-

ber²⁴, thus the precise spawning site may change with the location of the salinity front. In addition, because adults will arrive along a broad east-west longitude depending on their Asian origin, the spawning region may span several longitudinal degrees. But the salinity front provides an important hydrographic cue for spawning because it marks the North Equatorial Current which leads to the Kuroshio Current²⁵. These major currents provide a means for returning the leptocephali to the coastal waters in Eastern Asia²⁴ from which the adults originated (Fig. 2).

There are two important differences between the Atlantic and Pacific Ocean spawning sites. The northern limit of spawning in the Sargasso Sea is marked by a thermal front^{26,27}, whereas in the Pacific Ocean it is marked by a salinity front. This suggests that the physical trigger for eel spawning may differ. Comparing latitudes, eels breed at 25–30° N in the Atlantic Ocean²¹, whereas in the Pacific Ocean it is much further south, about 15° N. Thus latitude does not itself constrain spawning. Instead, the key similarity in both oceans is the position of the spawning areas relative to major currents, the Gulf Stream of the Atlantic Ocean²⁸ and the Kuroshio Current of the Pacific Ocean, that can return the offspring and thus complete the recruitment of the eels²⁴. Natural selection has apparently favoured a spawning location for eels in both oceans that enhances the transportation of leptocephali to their respective growth habitat. □

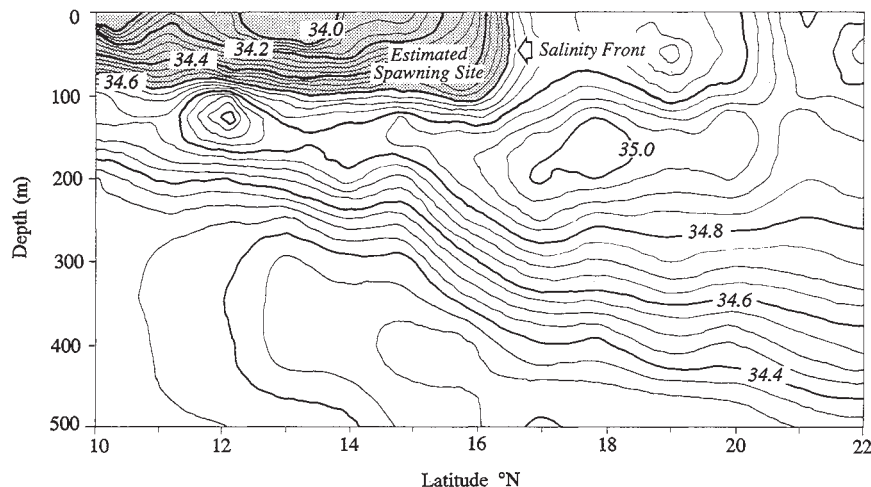


FIG. 4 The depth profile for salinity at the 137°E transect. Latitudinally, there is a discernible salinity front (arrow) at 16° N. Vertically, there is a halocline at 75–100 m deep. The low salinity surface water mass is shaded. In contrast to this major salinity gradient, there was very little change in water temperature. The estimated spawning site of the Japanese eel is shown. The depth profiles for salinity at the other transects showed mostly similar patterns to this 137°E transect with a discernible salinity front at 15–16° N, forming a broad east-west salinity front (S. Kimura *et al.*, unpublished results).

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The VASE exon downregulates the neurite growth-promoting activity of NCAM 140

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AXONAL growth, guidance and synapse formation are controlled by receptors on neuronal growth cones that can recognize positive and inhibitory cues in the local microenvironment^{1–3}. Four well characterized receptor systems are known that recognize the growth-promoting activities associated with the extracellular matrix and the membranes of cells such as astrocytes, muscle cells and Schwann cells; these are the integrins⁴ and the homophilically binding cell adhesion molecules neural-cell adhesion molecule (NCAM), N-cadherin and L1 (refs 5–12). Alternative splicing generates 20–30 isoforms of NCAM and these can also be differentially glycosylated¹³. There are two sites where alternative splicing changes the extracellular structure of membrane-bound NCAM and one of these (the MSD1 region) does not obviously affect function⁶. Here we report that the variable alternatively spliced exon (VASE) in immunoglobulin domain 4 downregulates the neurite outgrowth-promoting activity of NCAM. The high level of VASE expression in the adult central as compared with peripheral nervous system²¹ could contribute to the poor regenerative capacity of the former.

We transfected 3T3 fibroblasts with plasmid vectors containing complementary DNAs encoding NCAM 140 and NCAM 140-VASE and isolated stable clones²⁰. NCAM 140 and NCAM 140-VASE were expressed at the cell surface^{18,20} and

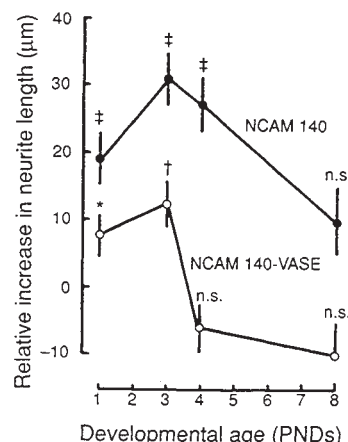


FIG. 1 Neurite outgrowth from cerebellar neurons cultured on monolayers of 3T3 cells expressing NCAM 140 (●) as compared with NCAM 140-VASE (○). Dissociated cerebellar neurons were cultured on confluent monolayers of control and transfected 3T3 cells before being fixed and stained for GAP43 immunoreactivity. The results show the mean length of the longest neurite per cell ($\pm$ s.e.m.) for between 120–180 individual neurons. Measurements are expressed as the absolute difference between growth on transfected as compared to parental 3T3 cells. Values measured in the latter were 32.0 ± 3.0 (185), 33.5 ± 2.2 (182), 48.9 ± 3.9 (141) and 64.8 ± 4.8 (124) for PND1, 3, 4 and 8 neurons respectively (mean $\pm$ s.e.m., n). The level of significance for the differences in means found between transfected and parental 3T3 cells is shown and was determined by the double-sided Student's t -test. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$; n.s., not significant.

METHODS. The cDNA for the 140K isoform of NCAM was prepared as described^{6,20}. The VASE region was inserted into the NCAM 140 expression construct by first removing a 373-base-pair (bp) *NcoI*-*BalI* fragment and replacing this with a similar 403-bp polymerase chain reaction-generated fragment that had been produced from VASE exon-expressing H69 tumour cells³⁰. Insertion of the VASE exon was confirmed by sequencing. Rat cerebellar neurons were isolated as a single cell suspension^{6,8}. Confluent monolayers of control and transfected 3T3 cells were established over a 24-h period in individual chambers of a 4-well tissue culture slide (Lab-Tek)^{6,8,10}. Co-cultures were established by adding $\sim 5,000$ cerebellar neurons to monolayer cultures; culture medium was SATO containing 2% FCS. After 15–24 h the cocultures were fixed with 4% paraformaldehyde, permeabilized with methanol (-20°C) and stained for GAP43 immunoreactivity⁸ using a rabbit polyclonal antibody (a gift from R. Curtis and G. Wilkin). The length of the longest neurite for each GAP43-positive neuron was determined by computer-assisted digitized image analysis^{8,10}.

ran as indistinguishable bands corresponding to a relative molecular mass ~ 140 K on gel electrophoresis (data not shown). Initial analysis compared neurite outgrowth over parental 3T3 cells with that found on two individual clones of transfected cells that expressed similar levels of either NCAM 140 or NCAM 140-VASE. Rat cerebellar neurons isolated at postnatal day (PND) 1, 3, 4 and 8 were cultured for 15 h on confluent monolayers of 3T3 cells before being fixed, and the average length of GAP-43 positive neurites determined^{6,8,10}. Expression of NCAM 140 was associated with a neurite growth-promoting response from neurons isolated at PND1, 3 and 4 (Fig. 1). There was only a residual response from PND8 neurons (Fig. 1). The enhanced neurite outgrowth response was inhibited by a monoclonal antibody raised against NCAM⁸. In contrast, PND1 and 3 neurons showed a reduced response to NCAM 140-VASE and those isolated at PND4 and 8 failed to show any response. A similar developmental loss of neuronal responsiveness to NCAM has been reported for chick retinal ganglion cells^{8,10} and rat hippocampal neurons (P.D. *et al.*, manuscript submitted). All these neurons remain highly responsive to chick N-cadherin transfected into and expressed by 3T3 cells, demonstrating that loss of responsiveness to NCAM is of developmental significance (ref. 10, and data not shown). PND6 and PND8 neurons tested in a 24-h coculture assay focused the

period of loss of neuronal responsiveness to this developmental window and confirmed the inability of PND6 neurons to respond to NCAM 140 containing the product of the VASE exon (Table 1). PND8 neurons showed a similar extent of growth on all three monolayers, suggesting that the ability of the monolayer to support non-CAM-dependent neurite outgrowth is similar. The binding of a monovalent iodinated Fab fraction of an anti-human NCAM monoclonal antibody demonstrated the presence of ~15% more NCAM on the surface of the VASE-expressing cell line (Table 1); thus the poor response is not attributable to reduced levels of NCAM expression.

To determine whether the differential responsiveness to NCAM 140 and NCAM 140-VASE reflected a qualitative change in NCAM function rather than quantitative differences in the level of expression of these isoforms, PND4 cerebellar neurons were cultured for ~20 h on a number of additional clones of transfected 3T3 cells that expressed differing levels of NCAM 140 or NCAM 140-VASE. The neurite outgrowth response was maximal over the range of NCAM 140 expression found on two additional clones (Fig. 2a). Three clones transfected with NCAM 140-VASE were all substantially less effective at inducing neurite outgrowth despite expressing lower, intermediate, and greater levels of NCAM at the cell surface (Fig. 2a).

Polysialic acid on neuronal NCAM can modulate NCAM but not N-cadherin or integrin dependent neurite outgrowth^{8,10}.

TABLE 1 Iodinated NCAM antibody binding to and neurite outgrowth over transfected cells

Monolayer	¹²⁵ I-labelled Fab binding (c.p.m. per 1,000 cells)	Mean neurite length (μm)	
		PND6	PND8
Parental 3T3	—	93.6 ± 6.4 (118)	110.0 ± 8.8 (104)
NCAM 140 transfectant	680 ± 27 (6)	185.6 ± 11.9 (97)*	109.9 ± 8.2 (108)†
VASE transfectant	785 ± 48 (6)	103.2 ± 6.7 (131)†	102.9 ± 7.2 (122)†

Neurite outgrowth from PND6 and PND8 neurons grown for 24 h on monolayers of control and transfected 3T3 cells was determined (Fig. 1). Each value is the mean ± standard error of the mean (s.e.m.) for the given number of neurons. Statistical analysis was in Fig. 1. A monovalent Fab fraction of the anti-NCAM monoclonal antibody (ERIC-1; ref. 29) was prepared and iodinated by the chloramine-T method. Individual batches of antibody were titrated against a cell line expressing high levels of human NCAM and were used at 2–3 fold greater concentration than that giving maximal binding. Under assay conditions there was a linear relationship between cell number (tested at up to 12,000 cells) and antibody binding. Antibody binding to untransfected cells was consistently 5–10% of the value measured against cells positive for human NCAM. About 2,000 cells from individual clones of transfected 3T3 cells were set up as 12 replicate cultures in individual wells of a polylysine-coated 96-well microtitre plate. After ~18 h, the binding of a saturating concentration of ¹²⁵I-labelled Fab ERIC-1 was determined by antibody incubation at 37 °C for 1 h, followed by four rapid washes with culture medium containing 10% FCS. Six cultures were solubilized with 0.1 M NaOH and ¹²⁵I-labelled Fab ERIC-1 binding was measured; six were fixed with methanol for determination of cell number⁶. Results show the mean binding of antibody ± s.e.m. corrected for variations in cell number, with the non-specific binding of antibody, determined against parental 3T3 cells, subtracted. The latter value was ~10% of the positive values. Similar results were obtained with two further batches of iodinated antibody and similar ratios of the binding of an independent monoclonal antibody determined by both enzyme-linked immunosorbent assay and by quantitative immunofluorescence (data not shown, and refs 6, 8). When specific activities of iodinated antibody were determined by trichloroacetic acid precipitation and competition binding assays performed against known concentrations of non-iodinated Fab, the above lines were calculated to bind ~1 × 10⁶ Fab molecules per cell, which suggests that a similar number of NCAM molecules are present.

* $P < 0.001$.

† Not significant.

Enzymatic removal of polysialic acid from the rat cerebellar granule cells inhibited (by 70–80%) the NCAM component of neurite outgrowth on the three clones of NCAM 140-transfected cells described here (shown for two of these in Fig. 2b). In contrast, removal of neuronal PSA did not affect the basal, presumably integrin-dependent^{8,10}, neurite outgrowth over parental and NCAM 140-VASE-expressing 3T3 cells (Fig. 2b). Thus, whereas VASE can inhibit the ability of NCAM to act as a substrate for neurite outgrowth, polysialic acid is an equally important determinant of the ability of neuronal NCAM to act as a receptor molecule that transduces the homophilic binding signal into a cellular response.

NCAM-dependent neurite outgrowth is probably a complex multistep process that initially involves homophilic binding and increased cell adhesion, but ultimately depends on the activation of neuronal second messenger pathways. There is no simple relationship between adhesion and more complex responses such as neurite outgrowth; for example, whereas polysialic acid is a negative modulator of adhesion^{22,23}, it is a positive modulator of neurite outgrowth¹⁰. PC12 pheochromocytoma cells respond to transfected NCAM 140 in a manner similar to primary neurons²⁴. Expression of transfected NCAM in 3T3 cells also increases their adhesive properties and this can readily be measured following inactivation of Ca²⁺-dependent adhesion systems²⁵. We have measured the ability of PC12 cells to adhere and extend neurites on monolayers expressing NCAM 140 or NCAM 140-VASE. The results confirm that VASE impairs neurite outgrowth, but show that VASE does not inhibit initial NCAM-dependent adhesion. In fact there was a slight but variable increase in adhesion. (P.D., unpublished observations).

NCAM- and N-cadherin-induced neurite outgrowth is additionally dependent on the activity of a pertussis-toxin-sensitive G protein and consequent opening of both L- and N-type calcium channels in neurons²⁴. We found that a combination of L- and N-type calcium channel antagonists completely

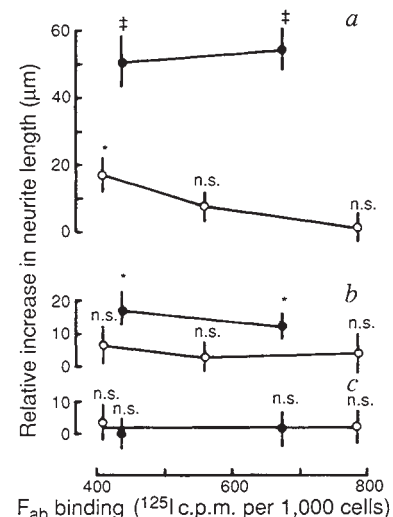


FIG. 2 The relationship between neurite outgrowth and NCAM expression at the cell surface. PND4 cerebellar neurons were cultured on two independent transfected clones of NCAM 140 (●) and three clones of NCAM 140-VASE (○)-expressing cells (including the clone described previously). The results show mean neurite lengths ± s.e.m. calculated from 120–180 neurons for cultures maintained in a, control media; b, media supplemented with a 1:250 dilution of endoneuraminidase N phage stock^{6,8}, or c, media supplemented with diltiazem (8 μM) and ω-conotoxin (0.16 μM)²⁴ as a function of the level of ¹²⁵I-labelled Fab ERIC-1 binding (the latter determined as in Table 1). Results on mean neurite length are expressed as the absolute difference found between growth on transfected as compared to parental 3T3 cells. Values measured in the latter co-cultures were a, 63.9 ± 4.2 (146), b, 67.2 ± 5.9 (121) and c, 70.2 ± 4.5 (157). The level of significance for the differences in means found between transfected and parental 3T3 cells is shown and was determined by the double-sided Student's *t*-test (see Fig. 1).

inhibits NCAM 140-dependent neurite outgrowth, but has no effect on neurite outgrowth over NCAM 140-VASE expressing cells or parental 3T3 cells (Fig. 2c). These data suggest that this signal transduction pathway is poorly activated in neurons following the binding of NCAM on neurons to VASE containing NCAM isoforms expressed on other cells.

The molecular basis for polysialic acid and VASE exerting modulatory effects on NCAM-dependent neurite outgrowth is unknown. Although a *trans*-interaction between NCAM molecules in neurons and NCAM in transfected cells is required for neurite outgrowth¹⁰, a *cis*-interaction between NCAM-NCAM in the same membrane and/or *trans/cis*-interactions between NCAM and other molecules^{26,27} could also contribute to the NCAM response. Polysialic acid and VASE could conceivably affect any of these interactions.

The amount of polysialic acid on NCAM can be correlated with plasticity in the developing and adult nervous system suggesting that it is a positive modulator of NCAM-dependent neurite outgrowth^{10,23,28}. Analysis of the expression of the VASE exon during development has led to the postulate²¹ that VASE could negatively modulate synaptic plasticity. For example, whereas ~3% of NCAM transcripts have VASE during early brain development, this progressively increases to ~50% of all transcripts in the adult central nervous system. In addition, VASE is not expressed in the adult olfactory epithelium, where neurogenesis and associated axonal growth continue throughout life. By demonstrating that VASE can directly inhibit the neurite outgrowth promoting activity of substrate-associated NCAM, these results provide the first direct evidence in support of this hypothesis.

VASE can also be expressed by neurons¹⁸ and this could alter the ability of NCAM to act as a transducing receptor in a way analogous to loss of polysialic acid. A combination of loss of polysialic acid and NCAM isoform switching has been proposed as a mechanism to explain the development loss of retinal ganglion cell responsiveness to substrate-associated NCAM¹⁰. The biological effects attributable to polysialic acid and VASE suggest that two independent mechanisms have evolved to control the neurite outgrowth promoting activity of NCAM. The reciprocal patterns of polysialic acid and VASE expression observed during development and in the adult central nervous system suggest that both may act to change the function of NCAM from a molecule that promotes plasticity (through second messenger pathways) to one that promotes stability (through direct adhesive interactions). □

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Specific low-affinity recognition of major histocompatibility complex plus peptide by soluble T-cell receptor

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THE T-cell receptor is necessary and sufficient for recognition of peptides presented by major histocompatibility complex molecules^{1,2}. Other adhesion molecules, like CD4 or CD8, play an auxiliary role in antigen recognition by T cells^{3,4}. Here we analyse T-cell receptor (TCR) binding using a soluble rather than a cell-bound receptor molecule. A TCR-immunoglobulin chimera is constructed with the variable and the first constant regions of both the TCR α - and β -chains linked to the immunoglobulin light-chain constant regions. This soluble TCR is expressed, assembled and secreted as an $\alpha\beta$ heterodimer by a myeloma cell line transfected with the recombinant genes. Furthermore, the soluble TCR is biologically active: it specifically inhibits antigen-dependent activation of the relevant T-cell clones and thus discriminates between proper and irrelevant peptides presented by major histocompatibility complex molecules.

The genes for the soluble T-cell receptor (sTCR) were derived from a mouse T helper (Th) hybridoma 14.3.d (derived from a T-cell clone V₂₋₁₅) that recognizes a haemagglutinin peptide of influenza virus A/PR/8/34 (H1N1) in the context of major histocompatibility complex (MHC) class II I-E^d (ref. 5). Variable regions of V₂₋₁₅ TCR genes are coded by V β 8.2J β 2.1 and V α 4J α 2B4 (ref. 6) and thus this TCR product can be detected with V β 8-specific monoclonal antibodies like KJ16 (ref. 7) and F23.1 (ref. 8). Phenotypically this clone has a high affinity because it is stimulated with low concentrations of antigen or peptide⁹ and because its stimulation is largely independent of the CD4 coreceptor (data not shown).

For the solubilization of the TCR (refs 10, 11), the variable region exons (V α J α and V β D β J β , respectively) and the first constant region exons (C α 1 and C β 1, respectively) of the α and β TCR chains were ligated to the constant region exon of the κ light chain (C κ) and introduced into eukaryotic expression vectors¹² (Fig. 1). The expression is therefore controlled by TCR α - and β -chain gene promoters and the C κ intron enhancer. The constructs were then introduced and expressed in the myeloma cell line J558L^{10,11}.

To study the expression and features of soluble TCR, intracellular samples and supernatants of transfected cells were subjected to immunoprecipitation, isoelectric focusing and subsequent immunoblotting (Fig. 2a). Intracellular expression of α and β chains in single (α or β) or double (α and β) transfection experiments was easily detected (Fig. 2a). In single transfection experiments, the β chains were secreted but the α chains were not (Fig. 2a). The $\alpha\beta$ double transfection experiments showed variability (examples of two clones are presented here). Both clones express the α and β chains (Fig. 2a; lanes 1, 2) and intracellularly they form $\alpha\beta$ heterodimers as shown by

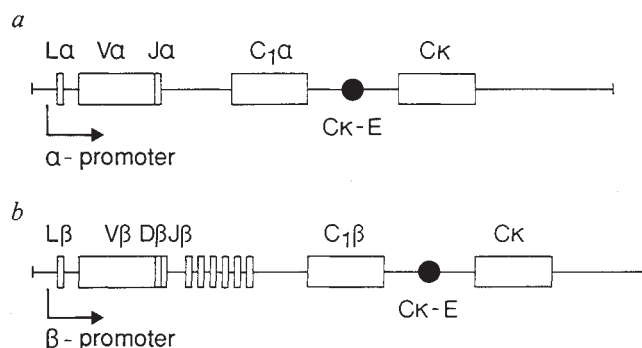


FIG. 1 Construction of genetically modified TCR chains. Portions containing exons coding for the soluble chimaeric TCR chains are shown (boxes, exons; lines, introns; filled circles, *C κ* enhancer). *a*, Plasmid pV α C1 α C κ ; *b*, Plasmid pV β C1 β C κ .

METHODS. *a*, Functionally rearranged *V α* region gene (*V α 4Ja2B4*; ref. 6, and our unpublished observation) was isolated from the *V α 2-15* genomic library. A subcloned 1.2-kb *Hind*III fragment containing the rearranged *V α* gene and its promoter region was inserted into expression vector pV α C1 α C κ (ref. 12). The *V κ* region was removed by *Bam*HI/*Sal*I digestion and the plasmid blunt-end ligated, generating the vector pV α C1 α C κ . *b*, An intermediary step was needed to construct the expression vector pV β C1 β C κ . The genomic fragment containing the rearranged *V β* promoter and gene (*V β 8.2J β 2.1*; ref. 6 and our unpublished observations) and first constant region exon of *C β 2* was inserted into pUC19 as a *Eco*RI/*Sac*I fragment. The final vector was created by ligating the *Eco*RI (blunt) 4.7-kb fragment from the pUC19/*C β 2* plasmid into expression vector p29.7cc3 (ref. 12), digested with *Xba*I/*Hind*III (blunt-ended). Vectors were introduced into J558L myeloma cells as described^{10,11}.

immunoprecipitation with F23.1 (Fig. 2*a*; lanes 9, 10). Secretion of the $\alpha\beta$ heterodimer varies from clone to clone: whereas some of the clones express, assemble and secrete the proper $\alpha\beta$ heterodimer (Fig. 2*a*; lanes 1, 5, 9, 13) others do not, even though they are able to secrete β homodimers (Fig. 2*a*; lanes 6, 14). The reasons for this phenomenon are not clear, but could explain in part previous failures to produce sTCRs by similar procedures (for review see refs 10, 13).

The secreted protein was next recovered from the cell culture supernatants by single-step affinity-column chromatography (Fig. 2*b*). The selection of high producers resulted in clones that secrete $\sim 10 \mu\text{g ml}^{-1}$ of sTCR, so several hundred milligrams of the pure protein can be obtained (Fig. 2*b*). The DEAE-Sephacel elution profile confirmed that most of the purified protein consists of $\alpha\beta$ heterodimers whereas $\beta\beta$ homodimers are present in small amounts (Fig. 2*c*). The fact that sTCR can be used as an immunogen to produce anti-clonotypic monoclonal antibodies which stain *V α 2-15* cells is consistent with 'solubilization' not causing major conformational changes in the protein structure (data not shown). In addition, these anti-clonotypic monoclonal antibodies react with >90% of the purified material in immunoprecipitation experiments. These results, in conjunction with the elution profile, suggest that functional $\alpha\beta$ heterodimers are being produced.

Direct binding assays with sTCR have failed so far. Therefore we have studied the biological activity of our sTCR preparation in cellular assays in which the inhibition of the antigen-specific activation of different T-cell hybridomas is measured (Fig. 3). For these assays, the T-cell hybridomas were stimulated with suboptimal concentrations of antigenic peptides presented by

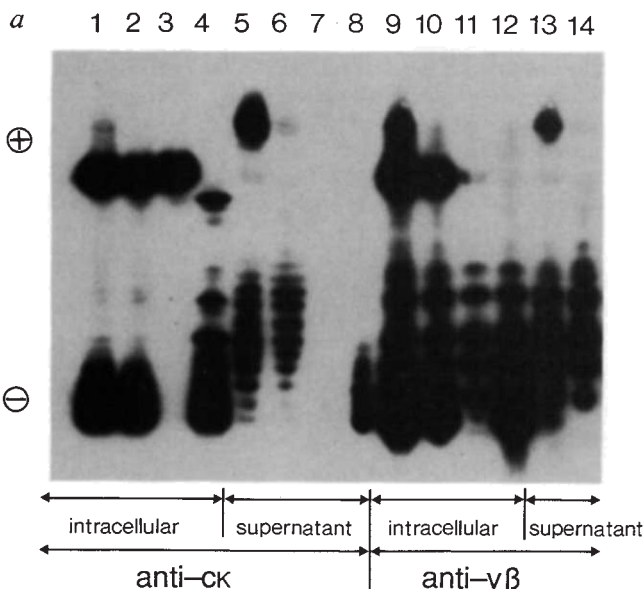
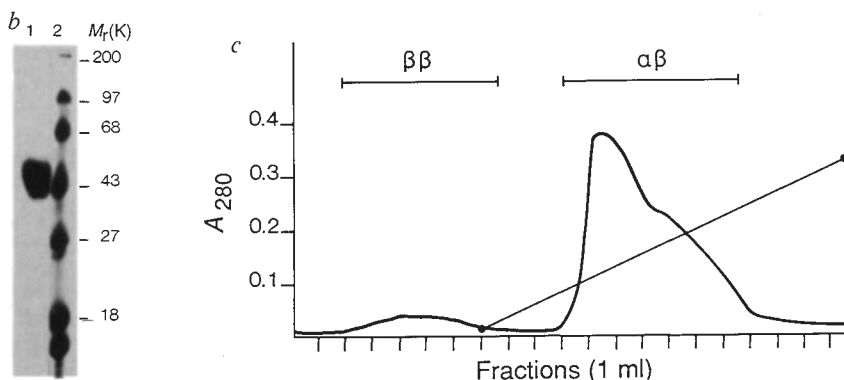


FIG. 2 Molecular characterization of soluble TCR. *a*, Isoelectric focusing (IEF) analysis of clones producing modified TCR chains. Four different clones were analysed: two produce TCR α and β chains (lanes 1, 2, 5, 6, 9, 10, 13, 14), one produces only α chains (lanes 3, 7, 11) and one only β chains (lanes 4, 8, 12). Clones were tested for intracellular and secreted chains. Cell lysates or culture supernatants were immunoprecipitated with either anti-C κ or anti-V β antibodies followed by IEF and immunoblotting. Alpha chains focus in acidic (+) and β chains in basic (-) regions of the IEF gel. Owing to the addition of sialic acids, secreted chains have a more acidic pI. As the immunoblot was developed with anti-C κ antibodies, lanes 9-14 contain background patterns of κ light chains originating from anti-V β antibodies (see lane 11 for background pattern). *b*, SDS-PAGE analysis of purified $\alpha\beta$ heterodimers. *c*, DEAE-Sephacel elution profile of the purified material.

METHODS. *a*, Cell lysates from 2×10^6 cells or 1.5-ml cell culture supernatants were immunoprecipitated with either anti-C κ (sheep anti-mouse C κ ; gift from E. Wagner) or anti-V β (F23.1) antibodies coupled to Sepharose beads. For IEF we used Ampholines pH 3.5-10 under reducing conditions in the presence of 8 M urea. To detect immunoblotted protein, rabbit anti-mouse C κ (gift from E. Wagner) and ¹²⁵I-labelled donkey anti-rabbit antibodies (Amersham) were used. *b*, sTCR heterodimers were purified from cell culture supernatants by affinity-column chromatography with a monoclonal antibody rat anti-mouse C κ (187.1)^{10,11}. Purified protein (30 μg) was analysed by SDS-PAGE (7%) under reducing conditions and stained with Coomassie blue. Relative molecular mass (*M_r*) markers are indicated on the right. *c*, Purified protein (2 mg) was loaded into a DEAE-Sephacel column in 25 mM phosphate buffer, pH 7.0. $\beta\beta$ homodimers, because of their basic pI, are in the flow-through. A linear salt gradient (0-500 mM NaCl) was used to elute $\alpha\beta$ heterodimers. Each fraction was tested on analytical IEF to identify protein peaks.



B lymphoma A20 cells¹⁴, in the absence or presence of sTCR, anti-MHC class II antibodies (14-4-4S)¹⁵ or their antigen-binding fragments (Fab). The specificity of sTCR was tested on the parental hybridoma 14.3.d and three other hybridomas, which share the specificity of the parental hybridoma towards the MHC/peptide complex in different ways (see Table 1). CD4⁺ hybridomas were used, and all of them express comparable levels of TCR.

The antigen-specific activation of all hybridomas was inhibited by the relevant anti-class II antibodies: for example, I-E^d-restricted hybridomas were inhibited by 14-4-4S monoclonal antibodies (Fig. 3a) and I-A^d-restricted hybridoma 3DO-54.8 (ref. 16) was inhibited by M5/114 (ref. 17; not shown). Complete antibodies were 50- to 100-fold better as inhibitors compared with their corresponding monovalent Fabs (only the 14-4-4S monoclonal antibody was tested). Soluble TCR is a much weaker inhibitor (Fig. 3b). Although the amounts of sTCR needed for 50% inhibition show a threefold variation between experiments (Fig. 3b shows an example of the most sensitive assay), the response of the different hybridomas follow similar patterns. Parental hybridoma 14.3.d and hybridoma LD3-8 (ref. 9), which share the same restriction element and peptide specificity (HA 110-120), could be inhibited by sTCR. However, Fabs of the 14-4-4S monoclonal antibody were ~1,000-fold better inhibitors than the sTCR.

Hybridoma 13.26.8 (ref. 18), which binds the same MHC molecule but has a different peptide specificity (sperm whale myoglobin), is inhibited by sTCR, although 10-fold less efficiently than hybridomas 14.3.d and LD3-8. This implies that

TABLE 1 Characteristics of the T-cell hybridomas

T-cell hybridoma	14.3.d	LD3-8	13.26.83	DO-54.8
CD4 expression	CD4 ⁺	CD4 ⁺	CD4 ⁺	CD4 ⁺
Restriction element	I-E ^d	I-E ^d	I-E ^d	I-A ^d
Antigen	influenza virus haemagglutinin	influenza virus	sperm whale myoglobin	chicken ovalbumin
TCR genes	v β 8.2 v α 4	v β 8.3 v α 2	v β 8.3 ?	v β 8 ?

?, Not known.

sTCR could have affinity for the MHC molecule in the absence of the specific peptide. The 14.3.d hybridoma is not stimulated by myoglobin/I-E^d complexes, and thus the supposedly weak affinity for class II alone is not sufficient to stimulate effector cells, indicating that only binding to the peptide and the MHC satisfies the affinity required for T-cell activation. The affinity towards class II alone could be important during positive selection in the thymus¹⁹, but more hybridomas have to be tested to confirm this.

sTCR has no effect on the I-A^d-restricted hybridoma 3DO-54.8 (ref. 16) and, hence, sTCR, like T-cell clones, can discriminate between I-A and I-E molecules. We were unable to inhibit the same hybridomas with sTCR when saturating amounts of peptide or suboptimal concentrations of anti-CD3 antibodies (145-2C-11)²⁰ were used to stimulate the responses (not shown). These controls exclude toxic nonspecific effects of sTCR, even at a final concentration of 3 mg ml⁻¹ (Fig. 3b).

Comparison of the concentrations needed for 50% inhibition of the hybridomas by the monovalent anti-class II antibody Fabs and sTCR provided an estimate of the affinity of sTCR for the I-E^d/peptide complex. The affinity of 14-4-4S Fab fragments was measured by Scatchard analysis (not shown) to be 10⁻⁸ M and thus the affinity of sTCR is estimated to be ~10⁻⁵ M, which corresponds to an antibody of very low affinity. It is unlikely that 14-4-4S recognizes the class II complex in the same way as TCR. This would make our affinity value an upper estimate. Alternatively, from the concentration of sTCR needed for 50% inhibition (Fig. 3b), we estimate the TCR dissociation constant (K_d) to be 5 × 10⁻⁶ M (average of four experiments). Similar results have been obtained using soluble MHC class II molecules²¹.

Antibodies and TCRs use different strategies to recognize antigens. Antibodies recognize soluble antigens and therefore need high-affinity antigen-binding sites, whereas TCRs are cell-surface molecules designed to recognize cell-bound antigens and presumably do not need high-affinity binding sites. To overcome the low affinity of TCR, T cells simultaneously use multiple TCRs to increase the avidity of recognition. This ensures that T cells are focused to appropriate targets; for example, TH cells 'help' only those B cells that have captured enough antigen and hence the specificity of B cell responses is enhanced. Similar kinds of arguments can also be applied for cytotoxic T cells. Interestingly, recent reports that about 100 antigenic complexes are needed to trigger activated T cells are consistent with this idea^{22,23}.

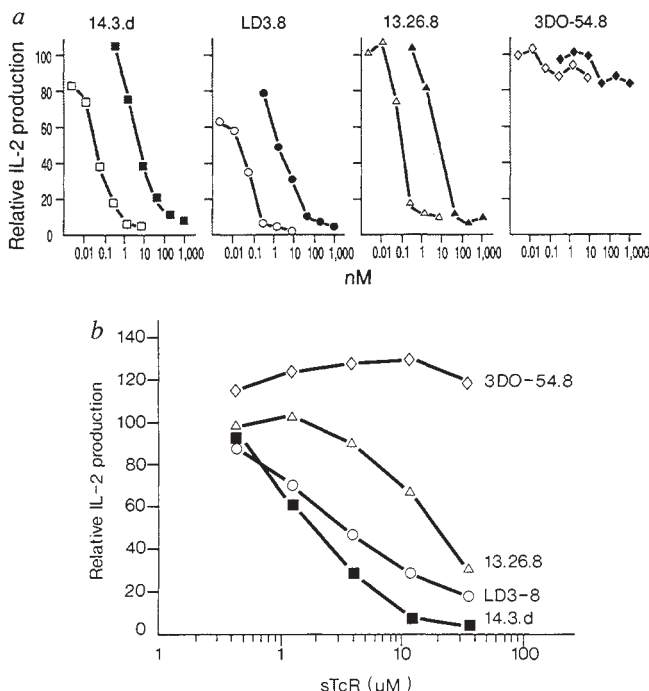


FIG. 3 Cellular inhibition assays. T-cell hybridomas were stimulated with suboptimal concentrations of peptides in the presence of A20 antigen presenting cells. T-cell activation was monitored by measuring interleukin-2 (IL-2) secretion. Stimulation of hybridomas is shown relative to stimulation without inhibitors (=100). a, Inhibition with anti-I-E^d antibodies (open symbols) or their Fabs (filled symbols) as indicated. b, Inhibition with sTCR as indicated.

METHODS. Irradiated A20 cells (5×10^4) were preincubated with peptides and titrated inhibitors for 2 h at 37 °C. T-cell hybridomas (5×10^4) were added and incubated for 20 h at 37 °C. Assays were done in triplicate in 96-well flat-bottom plates in a final volume of 200 μl. After incubation, collected supernatants were assayed for IL-2 by the IL-2-dependent cell line CTL-L. All assays were done in IMDM medium (Gibco) containing 5% fetal calf serum (Boehringer). There were 40,000–70,000 c.p.m. in uninhibited cultures and the background was ~300 c.p.m.

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MHC class II interaction with CD4 mediated by a region analogous to the MHC class I binding site for CD8

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INTERACTIONS between major histocompatibility complex (MHC) molecules and the CD4 or CD8 coreceptors have a major role in intrathymic T-cell selection¹. On mature T cells, each of these two glycoproteins is associated with a class-specific bias in MHC molecule recognition by the T-cell receptor. CD4⁺ T cells respond to antigen in association with MHC class II molecules and CD8⁺ T cells respond to antigen in association with MHC class I molecules. Physical interaction between the CD4/MHC class II molecules and CD8/MHC class I molecules has been demonstrated by cell adhesion assay^{2–5}, and a binding site for CD8 on class I has been identified^{6,7}. Here we demonstrate that a region of the MHC class II β -chain β_2 domain, structurally analogous to the CD8-binding loop in the MHC class I α_3 domain, is critical for function with both mouse and human CD4.

Two strategies were used to identify sites of MHC class II interaction with the CD4 coreceptor. Because mouse CD4 interacts poorly with human MHC class II molecules^{5,8}, and human CD4 interacts well with mouse class II molecules⁹, we substituted species-conserved residues in mouse MHC class II with the corresponding human residues and looked for selective decreases in interaction with mouse CD4. Second, because of the structural homologies^{10–14} and similar functions of MHC class I and class II molecules, we anticipated that CD4 might interact with class II at a site analogous to the CD8-binding site. Therefore, class II mutations in the region corresponding to residues 223–229 of the class I α_3 domain were examined for loss of interaction with CD4.

Mutated $\text{A}\beta^d$ complementary DNAs were cotransfected with wild-type $\text{A}\alpha^d$ cDNA into COS7 cells and those $\text{A}\beta$ cDNAs giving wild-type levels of transient expression were stably co-expressed with $\text{A}\alpha^d$ in mouse L-cells. To assess their structural integrity, the mutant $\text{A}\alpha^d\text{A}\beta^d$ molecules were examined using monoclonal antibodies against the peptide-binding domain (K24–199 for the α chain¹⁵ and MK-D6 (ref. 16) and M5/114 (ref. 17) for the β chain). Class II molecules displaying undiminished reactivity with all three antibodies were studied for interaction with CD4 using a functional assay involving T-cell activation by either peptide antigen or staphylococcal enterotoxin superantigen. T-cell hybridomas with different $\alpha\beta$ antigen receptors and expressing various coreceptor molecules (mouse or human CD4, mouse CD8, or none) were used. All produce lymphokines after receptor engagement by class II-associated ligands, but in the presence of an effective MHC class II–CD4

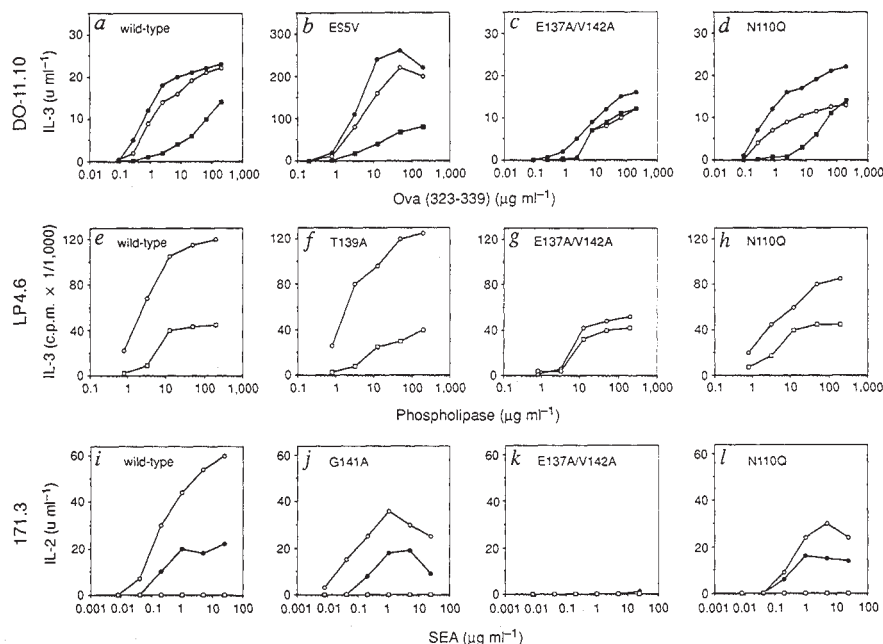
interaction, the antigen threshold is lower and the lymphokine production at a given antigen concentration higher. The major advantage of this assay is that it is internally controlled, in that a mutation in $\text{A}\beta^d$ selectively affecting CD4 binding to MHC class II will decrease the level of lymphokine production and increase the antigen threshold for the CD4⁺ hybridomas, but will not alter the dose response of the CD8⁺ or coreceptor-negative hybridomas.

The functional effects of the mutations, all located in the β_2 domain (Table 1), can be grouped into several categories, with a representative example of each shown in Fig. 1: no effect on CD4–class II interaction (Fig. 1*b, f, j*); decreased function with CD4 from either species (Fig. 1*c, g, k*); and decreased interaction with mouse but not with human CD4 (Fig. 1*d, h, l*). Substitution of alanine for glutamic acid at position 137 almost eliminated the contribution of mouse CD4 to T-cell activation and decreased human CD4 coreceptor function. Three other single alanine substitutions in the putative loop region (residues 137–143), Val 140, Gly 141 or Val 142, also caused decreases of varying degree in the contribution of CD4 to T-cell stimulation. A mutant $\text{A}\alpha\text{A}\beta$ molecule with two alanine substitutions in this region (E137A/V142A; Fig. 1*c, g, k*) did not elicit detectable CD4 coreceptor function. Stimulation of CD4⁺ T cells by this molecule was reduced to the level observed after stimulation of CD8⁺ or coreceptor-negative cells by either wild-type or E137A/V142A mutant molecules. These mutations had the same effect as removing CD4 from the responding T cell. These results are consistent with the mutations selectively impairing only the CD4-binding function of class II molecules. As the mutations changed wild-type residues to alanine, the loss of function appears to reflect elimination of a positive contribution to CD4 binding rather than introduction of a side chain with interfering activity (except possibly at Gly 141).

Substitution of asparagine at position 110 of $\text{A}\beta^d$ with glutamine, the residue occurring in most human alleles at this position, partially reduced the coreceptor function of murine CD4, but showed no effect on the responses of hybridomas expressing human CD4, murine CD8, or no coreceptor (Fig. 1*d, h, l*). Therefore, this residue probably contributes to the impaired interaction between murine CD4 and human class II (refs 5, 8). To explore further the structural basis of species-specificity in the MHC class II–CD4 interaction, Val 140 was changed to alanine and Ser 143 was replaced with valine. This generated a mutant $\text{A}\beta^d$ chain displaying all the human residues from positions 137–143, the region analogous to the CD8-binding loop of HLA-A2 (ref. 7). In contrast to the $\text{A}\beta^{\text{N110Q}}$ mutation, these multiple substitutions did not decrease the relative stimulation of the T-cell hybridoma expressing murine CD4 when compared to the CD8⁺ hybridoma, although they slightly enhanced the response of T cells bearing human CD4 (Fig. 2). Two additional mutations displayed a similar phenotype (Table 1), one of which also involved the change of Ser 143 to valine. The presence of valine at 143 could provide additional strength to the class II–CD4 interaction, accounting for the retention of function of the 140/143 double mutant possessing the otherwise detrimental Ala 140.

Our results identify the region of the β_2 domain between residues 137–143 as a primary determinant of class II–CD4 interaction. This conclusion is consistent with data independently obtained by Cammarota *et al.*¹⁶ using a peptide binding assay. On the basis of the crystal structure of the human class I molecule HLA-A2 (ref. 18), a model for the structure of the β_2 domain of $\text{A}\beta^d$ was generated (Fig. 3). In this model the side chain of Glu 137, which extends out from the first part of a loop in the β_2 domain that is analogous to the CD8-binding loop in the class I α_3 domain, is available for interaction with the positively charged residues in the region of CD4 that affects binding to class II (refs 19, 20). The lack of strong effects of single alanine substitutions other than at positions 137 and 140 could indicate that the individual contribution of any of these

FIG. 1 Antigen dose-response curves for sets of murine T-cell hybridomas (DO-11.10, *a-d*; LP4.6, *e-h*; 171.3, *i-l*) expressing either mouse (○) or human CD4 (●), mouse CD8 (■), or no coreceptor (□) after stimulation with antigen presented by wild-type or mutant $A\alpha^dA\beta^d$ transfected L-cells. METHODS. T-cell hybridomas were sorted into CD4⁺ and CD4⁻ populations by magnetic bead separation after staining with the monoclonal antibody, GK1.5. CD4⁻ DO-11.10 cells³⁰ were infected with defective retroviruses that contain the *neo^r* gene together with either mouse CD4, human CD4, or mouse CD8. cDNAs encoding mouse CD4 (ref. 31) or CD8 (ref. 32) were inserted into the retroviral vector pMNC (ref. 33). The ecotropic packaging cell line Ψ 2 was transfected with these constructs, and culture supernatants from transfected cells were used to infect the amphotropic helper cell line PA317 to generate virus-producing lines (R.N.G. *et al.*, unpublished observations). The defective retrovirus MNST4, containing human CD4 cDNA, was produced by the MNST4 DAMP (ref. 33) cell line. T hybridomas (5×10^4 cells) were stimulated by coinubation with 5×10^4 $A\alpha^dA\beta^d$ -expressing L-cells in the presence of various concentrations of antigen³⁴. DO-11.10 cells (*a-d*) were activated by a synthetic ovalbumin peptide, Ova (323-339); LP4.6 hybridomas (L. Phillips, unpublished observations; *e-h*) were stimulated by honey-bee phospholipase A₂ (Sigma); and 171.3 cells³⁵ (*i-l*) were cultured in the presence of staphylococcal enterotoxin A (Toxin Technology). Culture supernatants were tested for interleukin-2 (IL-2) or interleukin-3 (IL-3) by their ability to support the proliferation of the IL-2-dependent cell line, CTL-L, or the IL-3-dependent line FD.C/1. Lymphokine concentrations were determined³⁴ and are expressed as units ml⁻¹, except in the case of the LP4.6 cells, for which the results are shown as the proliferation of FD.C/1 cells in response to a sixfold dilution of culture supernatant. Similar data were obtained with



the CD4⁺/CD4⁻ sorted DO-11.10 hybridoma stimulated by either Ova (323-339) or staphylococcal enterotoxin B. Data are from a single experiment for each hybridoma and its derivatives, except in the case of *b*, which shows a representative example of a mutation (E95V) not affecting CD4 coreceptor function, taken from a different experiment in which cytokine release was about 10-fold more than in the experiment yielding the data in *a*, *c* and *d*. These data and the results summarized in Table 1 are representative of 2-5 experiments with each mutant and hybridoma set.

residues to the binding of CD4 is small. The presence of threonine rather than valine at position 140 of mouse $E\beta$ indicates that $E\alpha E\beta$ could have a weaker interaction with mouse CD4 than $A\alpha A\beta$. A decrease in human CD4⁺ T-cell stimulation by recombinant DR molecules containing the mouse $E\beta$ β_2 domain has been reported³⁷.

The model also shows Asn 110 exposed on the same face of the β_2 domain as Glu 137. Introduction of an additional methylene group into residue 110 could sterically impair class II interaction with mouse CD4 mediated by the functionally conserved loop region, whereas human CD4 might accommodate a longer side chain in this region or have a sufficiently greater affinity than mouse CD4 for class II, so that minor steric disturb-

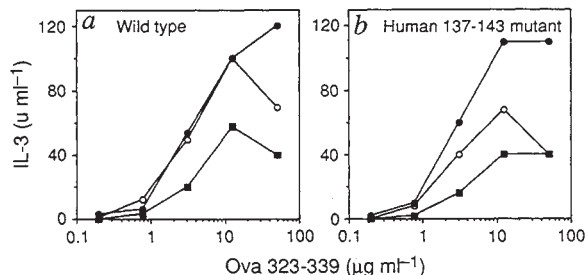


FIG. 2 Antigen dose-response curves for the DO-11.10 hybridomas stimulated with wild-type $A\alpha^dA\beta^d$ or a mutant $A\alpha^dA\beta^d$ carrying all human substitutions in the β_2 domain between residues 137 and 142. Assays are described in the legend to Fig. 1. Note that the transfected cells expressing the mutant MHC class II molecule had lower surface expression when used for this experiment (mean fluorescence intensity of wild type, 186; mutant, 112), resulting in a comparably decreased response by both the mouse CD4 and mouse CD8-expressing hybridoma cells relative to the response to the wild-type transfectant.

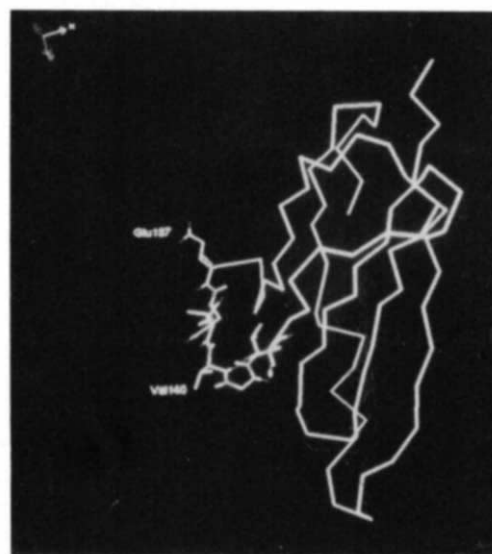


FIG. 3 Computer model of the β_2 domain of $A\beta^d$ generated using the programs QUANTA and CHARMM and the file 3hla (ref. 18) from the Brookhaven protein data base as a template. The residues are identified by their α -carbon atoms, except for residues 137-143, for which the side chains are also depicted. Residues Glu 137 and Val 140, the positions at which single alanine substitutions had the greatest effect, are labelled. The model is oriented so that the peptide-binding domain would be located above this β_2 domain, and CD4 would presumably approach the loop formed by residues 137-143 from above and left.

TABLE 1 Relative coreceptor function of mouse and human CD4 in response to mutant $\text{A}\alpha^d\text{A}\beta^d$ molecules

	Function with mouse CD4	Function with human CD4	HLA-A2 position/mutation	Function with human CD8
(1) E95V	+++	+++	R181	ND
(2) N110Q	+	+++	D196	ND
(3) R133L	+++	ND	R219	ND
(4) E137A	—	+	D223A	+
(5) E138A	+++	+++	Q224A	+++
(6) T139A	+++	++++	T225A	+++
(7) V140A	+	—	Q226A	—
(8) G141A	++	++	D227A	+
(9) V142A	++	+++	T228A	—
(10) S143A	+++	+++	E229A	+
(11) S143V	+++	++++	E229	ND
(12) Q146A	+++	++	E232A	+++
(13) L147A	+++	ND	T233A	+
(14) E137A/V142A	—	—	D223/T228	ND
(15) V140A/S143V	+++	++++	Q226/E229	ND

Numbering of residues is according to Kabat *et al.*²². Mutations are named after original residue followed by position and the new residue; ND, not done. A quantitative evaluation of CD4 coreceptor function with each mutant was derived from dose-response curves (Fig. 1) by normalizing responses of coreceptor positive and negative cells to the responses of the same T cells to wild-type $\text{A}\alpha^d\text{A}\beta^d$. This was done to control for differences in T-cell stimulation either due to variations in MHC class II expression on the transfected cells or in intrinsic responsiveness of the T hybridomas. A factor f , defined as the ratio between the concentration of antigen necessary to stimulate half-maximally the CD4-T-cell hybridoma and the antigen concentration necessary to cause equal lymphokine production by the CD4⁺ T hybridoma was determined for the wild-type $\text{A}\alpha^d\text{A}\beta^d$ and the mutant $\text{A}\alpha^d\text{A}\beta^d$ transfected L-cells. The fractional CD4 coreceptor function was defined as $F = (\log f (\text{mutant } \text{A}\alpha^d\text{A}\beta^d)) / \log f (\text{wild-type } \text{A}\alpha^d\text{A}\beta^d)$. A summary rating derived from several independent experiments was expressed as follows: $F < 0.25$ (—, no remaining coreceptor function); $0.25 < F < 0.5$ (+); $0.5 < F < 0.75$ (++); $0.75 < F < 1.5$ (+++), wild-type like); $F > 1.5$ (++++, significantly increased coreceptor function). Estimates of human CD8 coreceptor function are derived from Fig. 1 of ref. 7. The cDNAs encoding $\text{A}\alpha^d$ and $\text{A}\beta^d$ were in the eukaryotic expression vector, pcEXV-3 (ref. 23). Mutations 1–3, 8 and 12–14 were generated by the gapped heteroduplex method²⁴. To generate the gapped heteroduplex, pcEXV-3 was linearized with *EcoRI*, and pcEXV- $\text{A}\beta^d$ with *PvuII*. The two linear DNAs were mixed in equimolar amounts, denatured, and reannealed. The heteroduplex-containing mixtures were then incubated with a homologous oligodeoxynucleotide containing the mismatches necessary for the desired mutation. The sequences of the oligodeoxynucleotides were: 1, CGGCTTGACAGCCCAAT; 2, AGGCCCTCC-AACACCACA; 3, GCTGGTCTTGAATGGCC; 8, AGACAGTGGCGTCTCAT; 12, TCATCCACAGCGCTTATT; 13, TCCACACAGGCTATTAGG; 14, ATGGCCAGGCGGAGACAG and ACAGTGGGGGCGCTCATCC. Alternatively, the polymerase-chain reaction (PCR) and splicing by overlap extension modified from Ho *et al.*²⁵ were used. PCR was done with 20–500 ng template DNA (pcEXV- $\text{A}\beta^d$) and 1 μM of each primer using the GeneAmp kit (Perkin Elmer Cetus). The two invariant oligodeoxynucleotide primers were homologous to sequences of pcEXV-3 flanking the *EcoRI* insertion site: CCTGTACGGAAGTGTTACTT and ATCAATGTATCTTATCATGT. The sequences of the corresponding oligodeoxynucleotides carrying mismatches to generate the mutations were: 4, TCTCCGCTGGCCATTCC and ATGGCCAGGCGG-AGACAG; 5, CTGTTGCCTCTGGCCAT and GCCAGGAGGCAACAGTGG; 6, CACTGCTTCTCTCTGGCC and CCAGGAGGAAGCAGTGGG; 7, CCCCCGCTGTCTCTCTCT and AGGAGACAGCGGGGTCTCT; 9, TGAGGCCCTACTGTCTC and ACAGTGGGGGCGCTCATCC; 10, GGATGCGACCCCACTGT and GTGGGGTGCATCCACA; 11, GGATAC-GACCCCACTGT and GTGGGGTGCATCCACA; and 15, ACAGCGGGGTCTGTATCCACA and TACGACCCCGCTGTCTCTCT. PCR conditions: 1 min at 94 °C; 1 min at 37 °C; 1 min at 50 °C; 1 min at 72 °C (this step was extended for 5 s after each cycle) for 25 cycles in a DNA Thermal Cycler (Perkin Elmer Cetus). Products were gel-purified and 10 μL of each fragment were combined with the two external oligonucleotide primers for splicing by overlap extension. Conditions were as for the PCR except for the second step, which was at 54 °C for 2 min. Products from the splicing reaction were extracted with phenol and chloroform and digested with proteinase K (ref. 26). After re-extraction and precipitation with ethanol, they were incubated with 20 units of *EcoRI* for 2 h, isolated using the GeneClean kit (Bio101), and inserted into pcEXV-3. DNA was sequenced from double-stranded template using the Multiwell sequencing kit (Amersham). Mutant $\text{A}\beta^d$ DNA was cotransfected with wild-type $\text{A}\alpha^d$ DNA into COS7 cells²⁷ and transient expression was determined by FACS after staining with the M5/114 monoclonal antibody. Mouse L-cells were transfected with 5 μg $\text{A}\alpha^d$, 5 μg $\text{A}\beta^d$, and 0.4 μg pMo-neo using calcium phosphate coprecipitation²⁸. Populations of cells expressing comparably high levels of $\text{A}\alpha^d\text{A}\beta^d$ were selected, after staining with M5/114, by magnetic bead sorting on sheep anti-rat IgG-coated Dynabeads M-450 (Dyna). Structural integrity of the expressed $\text{A}\alpha^d\text{A}\beta^d$ heterodimers was determined by antibody dilution analysis²⁹ with the monoclonal antibodies K24–199 (ref. 15), MK-D6 (ref. 16), and M5/114 (ref. 17).

ances would not prevent functional activity. Evidence for this latter possibility stems from the generally stronger effects of single alanine substitutions on mouse CD4 coreceptor function (Table 1).

Our data do not exclude the existence of other important sites of CD4 interaction. Chimaeric MHC molecules containing only the class II β_1 domain interact with T cells whose function can be blocked by anti-CD4 antibody²¹; whether this indicates the existence of a direct interaction between CD4 and this domain of class II, and whether there might be a role of the α chain in coreceptor binding is unknown. □

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Identification of a CD4 binding site on the β_2 domain of HLA-DR molecules

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THE CD4 and CD8 molecules are transmembrane glycoproteins expressed by functionally distinct subsets of mature T cells. CD4⁺ and CD8⁺ T cells recognize antigens on major histocompatibility complex (MHC) class II-bearing and class I-bearing target cells respectively¹⁻³. The ability of monoclonal antibodies against CD4 and CD8 to block antigen recognition by T cells, as well as cell-cell adhesion assays⁴⁻⁷, indicate that CD4 and CD8 bind to non-polymorphic determinants of class II or class I MHC. Here we demonstrate that soluble recombinant HLA-DR4 molecules from insect cells and HLA-DR-derived peptides bind to immobilized

recombinant soluble CD4. CD4 binds recombinant soluble DR4 heterodimers, as well as the soluble DR4- β chain alone. Furthermore, two out of twelve DR4- β peptides could interact specifically with CD4. These findings show that CD4 interacts with a region of MHC class II molecules analogous to a previously identified loop in class I MHC proteins that binds CD8 (refs 8, 9).

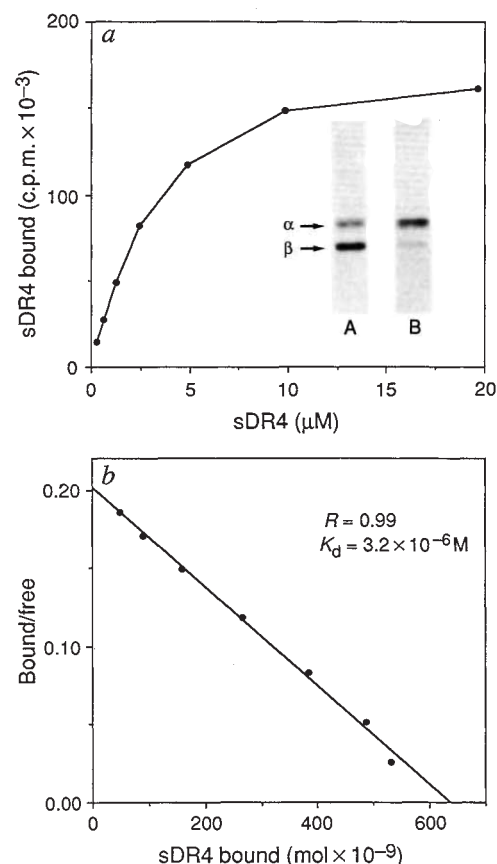
To establish the structural basis of the CD4-class II MHC interaction that could lead to cell-cell contact and influence T-cell signal transduction¹⁰⁻¹², we have developed an *in vitro* assay to measure specific binding of soluble recombinant CD4 to soluble HLA-DR molecules.

To generate a soluble form of the HLA-DR4 molecule, thus avoiding nonspecific interactions with-CD4 and the hydrophobic transmembrane region of HLA-DR, glycosylphosphatidylinositol-linked α and β heterodimers of the human MHC class II DR4Dw4 molecule were expressed in *Spodoptera frugiperda* (Sf9) insect cells.

DR4 heterodimers cleaved by phosphatidylinositol-specific phospholipase C (PLC) (Fig. 1) were isolated from the supernatants of transfected Sf9 cells by immunoaffinity chromatography using the monoclonal antibody L243 (ref 13). The isolated DR4 molecules ran as two bands (DR α and β) on SDS-PAGE (Fig. 1a). The soluble DR4 (sDR4) molecules produced in insect cells have functional and biochemical properties comparable to native HLA-DR4 molecules purified from a human DR4-homozygous lymphoblastoid cell line (A.S. *et al.*, manuscript submitted).

FIG. 1 Binding of soluble DR4 to immobilized sCD4. sCD4 (Hoffmann-La Roche and Genentech) was coupled to CNBr-activated Sepharose 4B (1 mg protein per ml of settled gel) as described¹⁶; the CD4-coupled Sepharose matrix was diluted 1:10 with Sepharose 4B and used for binding assays. DR4 was iodinated to a specific activity of 6.3×10^6 c.p.m. per μ g protein and mixed with unlabelled carrier DR4 to obtain a final specific activity of 23.5×10^3 c.p.m. μ g⁻¹. Increasing amounts of ¹²⁵I-labelled DR4 were incubated in the presence of 100 μ l of the CD4-coupled Sepharose matrix in a 15 mM phosphate buffer, pH 7.2, containing 150 mM NaCl and 0.5% NP40 (final volume 200 μ l). The amount of labelled material bound was determined as before¹⁶. The background of the experiment was estimated by measuring the binding of labelled DR4 to uncoupled Sepharose 4B in the same conditions. Less than 5% of the bound activity was retained by uncoupled Sepharose 4B. The saturation curve observed (a) was also plotted for Scatchard analysis (b); the dissociation constant (K_D) was obtained from slope $m = -1/K_D$. Insert in a: Purified sDR4 subjected to SDS-PAGE (15% polyacrylamide gel) under non-reducing conditions. Lane A, polyacrylamide gel after Coomassie blue staining; lane B, autoradiograph after immunoblotting with rabbit anti-DR1 polyclonal antiserum and iodinated protein G.

METHODS. The cDNAs encoding the extracellular domains of DR α and DR4 β were amplified by polymerase chain reaction (PCR). The oligonucleotides introduced an *EcoRI* site at the 5' end of each DR chain, as well as 22 nucleotides of overlapping DAF (decay accelerating factor) sequences at the 3' end of the sequence coding for the connecting peptide. 196 nucleotides of DAF cDNA were amplified using an oligonucleotide starting at amino acid 37 from the C terminus of DAF in combination with an oligonucleotide introducing an *EcoRI* site in the 3' untranslated region. The sequences were amplified, gel-purified and processed in the next PCR step. DR α and DR β fragments were each fused to the amplified DAF using PCR. Resulting fragments were subcloned into M13mp19 and completely sequenced. DR α -DAF and DR β -DAF genes were then cloned into the *EcoRI* site of pNR-704 (R. Gentz, manuscript submitted). These constructs were introduced separately into the genome of the *Autographica californica* nuclear polyhedrosis (AcNP) virus by homologous recombination. Sf9 cells were grown at 27 °C in EX-CELL 400 medium (JR Scientific) containing 1.5% FBS. Cell culture in spinner flasks and viral infection were carried out essentially as described²⁴. The recombinant viruses were purified by limiting dilution in microtitre plates followed by dot-blot hybridization with specific DNA probes. Infected Sf9 cells expressing DR4 heterodimers on their surface were harvested 60 h after infection, washed and resuspended in 70 mM ethanolamine, 10 mM EDTA, 0.1% *n*-octylglucoside, pH 7.2. Phosphatidyl inositol-specific phospholipase C (2 U per 10^9 cells) was used for an incubation of 4 h at 27 °C. DR4 molecules cleaved with phospholipase C were purified from the extracellular medium by immunoaffinity chromatography as described²⁵. DR4 was eluted from a column of L243 antibody using 50 mM diethylamine-HCl, pH 11.5, 1% *n*-octylglucoside, 0.15 M NaCl, 1 mM EDTA, 1 mM EGTA, 10%



glycerol, 0.03% NaN₃ and 10 mM freshly added iodoacetamide. Fractions (2 ml) were collected into tubes that contained 250 μ l 1 M Tris-HCl, pH 6.8, 0.15 M NaCl and 1% *n*-octylglucoside. Aliquots from each fraction were analysed by SDS-PAGE (a). Protein was quantitated using Coomassie protein assay reagent (Pierce) according to the manufacturer's instructions. Fractions containing most of the DR antigens were pooled, aliquoted and frozen at -70 °C.

The immunoaffinity-purified soluble DR4 molecules were radiolabelled to determine whether they could interact with CD4. It was found that binding to immobilized recombinant soluble CD4 was concentration-dependent, the interaction being rapidly completed within 30 min. Binding was specific as it was competed out by unlabelled purified DR4. To confirm the specificity of our assay, we investigated the effects of monoclonal antibodies directed against the CD4 molecule. As shown in Table 1, binding of sDR4 to soluble CD4 (sCD4) is inhibited by monoclonal antibodies (T151, 10A12.1, B66 and B53) against the V1-V2 domains of CD4 but is unaffected by OKT4, an antibody against an epitope on the membrane proximal half of the external region of CD4, or by 5b4 and W6/32 antibodies against urokinase or class I MHC molecules respectively. In binding experiments at equilibrium (Fig. 1a), DR4 showed saturable binding to immobilized CD4. Scatchard analysis of these data gave a K_d of 3.2×10^{-6} M (Fig. 1b). Soluble DR4 molecules also bound specifically to a CD4⁺ but not to a CD8⁺ T-cell clone (data not shown). Although less pronounced than the binding to sCD4, the binding to the CD4⁺ cells was specific as it was competed out by B53 antibody but not by OKT4.

To determine whether the DR β chain alone was able to interact specifically with the CD4 molecule, we infected Sf9 cells with baculoviruses carrying the hybrid DR4 β -DAF gene. No surface expression could be detected with an anti-DR β monoclonal antibody (1-1C4). In contrast, DR β subunits could

be detected on western blots of the cell lysate (Fig. 2a). DR β molecules were isolated from detergent-solubilized Sf9 cells by applying the detergent extract to a column of 1-1C4 antibody. Western blot analysis of the immunoaffinity-purified DR β chain revealed a single band. After cleavage with PLC, DR β molecules were reappplied to the 1-1C4 column, iodinated and used in the CD4 binding assay. Binding was concentration-dependent and was specifically inhibited by an excess of cold sDR4 as well as by the antibody B53 directed against the CD4 V1-V2 domains (Fig. 2b). Antibody OKT4, which binds outside the V1 and V2 domains of CD4, did not inhibit the binding reaction. We conclude that CD4 can interact with the DR β chain alone. Whether it can also interact with the DR α chain remains to be established.

Unlike class I, there is no detailed structural knowledge of a class II molecule. But indirect physical evidence¹⁴ and sequence homology¹⁵ indicate that class I and class II molecules have similar tertiary structures. As CD8 binds to the α_3 domain of class I (refs 8, 9) and the HLA-A α_3 domain shares 26% residue identity without gaps with the HLA-DR β_2 domain, CD4 should interact with the β_2 domain of class II. We therefore synthesized a series of overlapping peptides corresponding to the sequences of the DR β_2 domain. Equimolar amounts of each peptide were loaded onto the sCD4 column which was extensively washed to eliminate all unbound molecules; the retained material was eluted as described in the legend to Fig. 3 and the amount of

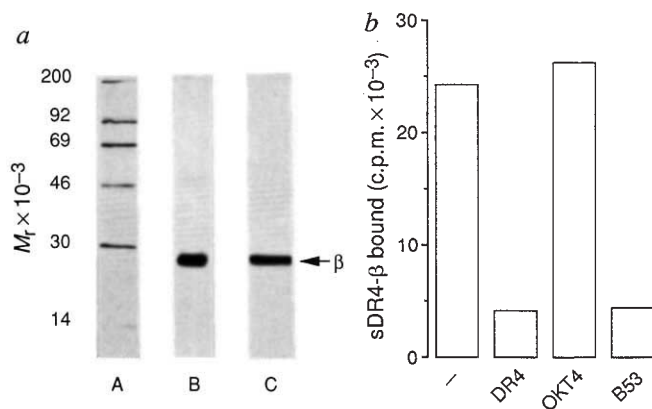


FIG. 2 Binding of soluble DR4- β chain to immobilized sCD4. *a*, Immunoblot analysis of soluble DR β chain. Lane A, M_r markers stained with Coomassie blue; lane B, cell lysate of Sf9 insect cells, infected with baculoviruses carrying the hybrid DR4 β -DAF gene, was analysed by immunoblotting using the 1-1C4 monoclonal antibody (Roche) directed against the DR β chain; lane C, immunoblot of immunopurified DR β molecules. *b*, The binding of the purified sDR4- β chain to CD4-coupled Sepharose was determined as described in Fig. 1 legend, except that 1×10^5 c.p.m. 125 I-labelled DR4 β (specific activity 4.5×10^6 c.p.m. μg^{-1}) were used. Competition experiments were done as described (Table 1 legend) using $50 \mu\text{g ml}^{-1}$ of sDR4 and either B53 or OKT4 monoclonal antibodies to inhibit the binding of the β chain to the resin. The background binding of the 125 I-labelled β chain to uncoupled Sepharose 4B was subtracted.

METHODS. Sf9 cells were infected with recombinant baculoviruses carrying the hybrid DR4 β -DAF gene as described (Fig. 1 legend). DR4 β molecules were extracted 72 h postinfection from pelleted Sf9 cells in lysis buffer: 50 mM Tris-HCl, pH 8, 2% deoxycholate, 1% NP-40, 10 mM iodoacetamide, 1 mM MgSO_4 , 20 $\mu\text{g ml}^{-1}$ DNase, 1 mM PMSF and 10 $\mu\text{g ml}^{-1}$ of each soybean trypsin inhibitor, antipain, pepstatin, leupeptin and chymostatin. The extract was stirred on ice for 60 min, EDTA was added to 5 mM and centrifuged at 100,000 g for 60 min. The supernatant fraction was incubated with 5 ml of CNBr-Sepharose 4B-immobilized 1-1C4 antibody (8 mg per ml gel) for 3 h at 4 °C. The gel was transferred into a column and washed. DR β antigen was eluted with 50 mM diethylamine-HCl, pH 11.5, 0.15 M NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% octyl- β -D-glucopyranoside, 10 mM iodoacetamide, 0.03% NaN_3 . Fractions (2 ml) were collected into tubes that contained 250 μl 1 M Tris-HCl, pH 6.8, 0.15 M NaCl and 1% octyl- β -D-glucopyranoside. After cleavage with phospholipase C, DR β molecules were immunoaffinity-purified, iodinated and used in the CD4 binding assay.

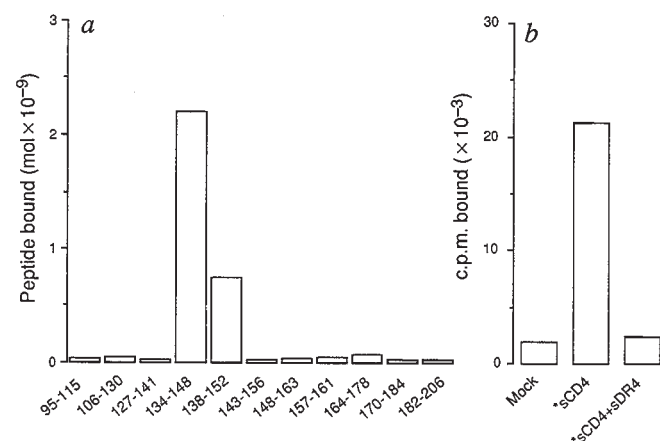


FIG. 3 Binding of DR β_2 -derived peptides to CD4. Peptides were synthesized with a MilliGen model 9050 peptide synthesizer using Fmoc chemistry; they were purified by preparative reversed-phase HPLC and homogeneity of each peptide confirmed by analytical reversed-phase HPLC. Characterization was performed by fast atomic bombardment-mass spectroscopy and the expected molecular ions were observed. The binding of DR β_2 -derived peptides to sCD4 (*a*) was determined by affinity chromatography. Briefly, 1 ml of a 650 μM solution of a given peptide in phosphate buffer, pH 7.2, was filtered through a column of 1.2 ml uncoupled Sepharose 4B to remove any particulate or insoluble material. The resulting flow-through was repeatedly passed through a column of 1.2 ml of CD4-coupled Sepharose (1 mg of sCD4 per ml of activated Sepharose slurry) at room temperature. After loading, the column was washed extensively with equilibration buffer until the absorbance reached the baseline at 222 nm, then eluted with a solution containing 150 mM NaCl and 1 mM HCl, pH 3; the amount of eluted peptide was determined spectrophotometrically. As a control, non-activated Sepharose 4B or Sepharose 4B coupled to unrelated proteins was used to determine nonspecific binding of peptides to the matrix; no binding was observed for all peptides in *a*. To test by competition experiments the specificity of the interaction of DR-derived peptides with sCD4, the binding of 125 I-labelled sCD4 (specific activity 5×10^6 c.p.m. μg^{-1}) to an affinity column obtained by coupling peptide 134-148 to activated carboxyhexyl-Sepharose 4B was tested (*b*). The affinity matrix (0.5 mg peptide per ml of CNBr-activated resin) was prepared according to ref. 16. Binding of sCD4 aliquots (50×10^3) was determined as described in Fig. 1 legend; inhibition of this binding by excess unlabelled DR4 (10 $\mu\text{g ml}^{-1}$ final concentration) was also measured; as a control the binding of sCD4 to uncoupled carboxyhexyl-Sepharose 4B was tested.

TABLE 1 Specificity of binding of ^{125}I -labelled sDR4 to sCD4

Antibody	Specificity	DR4 bound (mean c.p.m. $\pm$ s.d.)
OKT4	CD4	39,648 $\pm$ 2,535
T151	CD4 (V1-V2)	5,040 $\pm$ 394
10A.12	CD4 (V1-V2)	2,816 $\pm$ 165
B66	CD4 (V1-V2)	3,600 $\pm$ 251
B53	CD4 (V1-V2)	2,080 $\pm$ 174
W6/32	HLA-A,B,C	33,748 $\pm$ 1,849
5B4	Urokinase	35,952 $\pm$ 3,015

The binding of ^{125}I -labelled sDR4 (1×10^5 c.p.m.) to CD4-coupled Sepharose was measured as described (Fig. 1 legend). To test the ability of various monoclonal antibodies against CD4 to inhibit this binding, 0.1 mg ml^{-1} CD4-coupled Sepharose resin were preincubated for 1 h at room temperature in the presence of each antibody ($50 \mu\text{g ml}^{-1}$) in phosphate buffer, pH 7.2, containing NP-40 0.5% and 3% glycerol before exposure to labelled sDR4 (final volume 200 μl). After incubation the resin was repeatedly washed and the amount of material bound in the presence or in the absence of competing antibodies was determined according to ref. 16. The anti-CD4 monoclonal antibodies used were: OKT4 (ref. 17), T151 (ref. 18), 10A12 (ref. 19), B66 (ref. 20) and B53 (ref. 21). Monoclonal antibodies directed against human MHC class I determinants, W6/32 (ref. 22) and anti-urokinase 5B4 (ref. 23) were used as controls.

bound peptide measured spectrophotometrically. We found that peptide 134-148 (NGQEEKAGVVSTGLI), and to a lesser extent peptide 138-152 (EKAGVVSTGLIQNGD), were specifically adsorbed to sCD4-coupled Sepharose 4B (Fig. 3a), but not to the uncoupled Sepharose 4B (data not shown). The other peptides were not retained on the affinity matrix. To characterize further the CD4-DR β peptide interaction, peptide DR β (134-148) was immobilized on the Sepharose column and used to measure the interaction with ^{125}I -labelled sCD4. Labelled CD4 bound to peptide coupled but not to uncoupled Sepharose-4B. This binding could be completely inhibited by an excess of cold sDR4 or B53 antibody, whereas the antibody OKT4 was not inhibitory. These results indicate that the interaction of CD4 with class II molecules involves specific residues on the β_2 domain; also they are consistent with results independently obtained by R. König *et al.* (manuscript submitted) using site-directed mutagenesis.

The fact that peptide 138-152 is considerably less potent than peptide 134-148 in binding CD4 argues for the direct involvement of the residues on the N terminus of the 134-148 peptide in binding CD4. This region of MHC class II molecules is highly homologous to a sequence on an exposed loop of the α_3 domain of HLA-class I (residues 223-229) that is a major contact site with the CD8 α chain⁸. These results indicate that CD4 may interact with class II molecules in a manner similar to the class I-CD8 interaction. □

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Nuclear factor of activated T cells contains Fos and Jun

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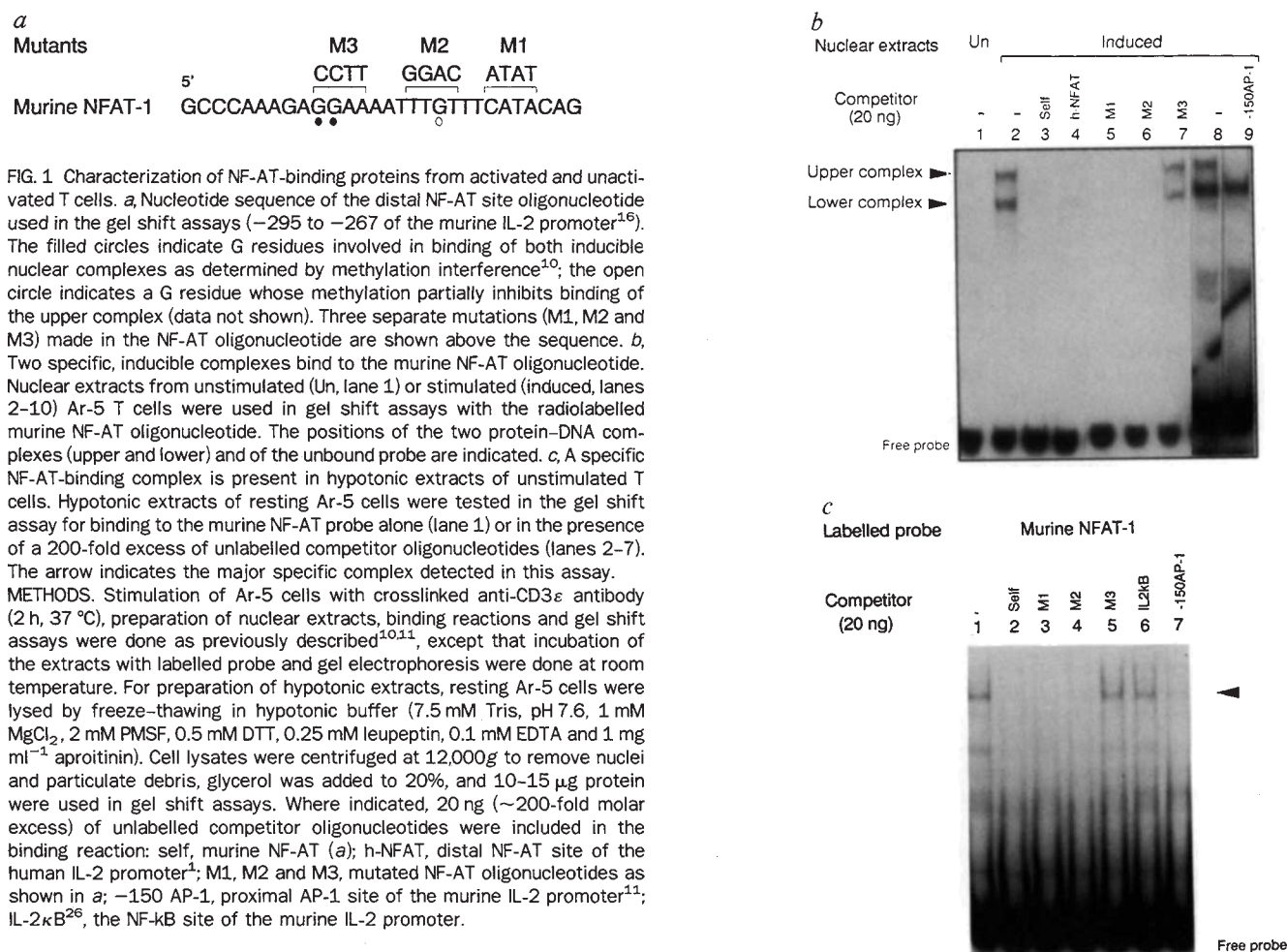
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THE nuclear factor NF-AT (ref. 1) is induced in T cells stimulated through the T-cell receptor/CD3 complex, and is required for interleukin-2 (IL-2) gene induction. Although NF-AT has not been cloned or purified, there is evidence that it is a major target for immunosuppression by cyclosporin A (CsA) and FK506 (refs 2-7). NF-AT induction may require two activation-dependent events: the CsA-sensitive translocation of a pre-existing component and the CsA-resistant synthesis of a nuclear component⁸. Here we report that the newly synthesized nuclear component of NF-AT is the transcription factor AP-1. We show that the inducible nuclear form of NF-AT contains Fos and Jun proteins. Furthermore, we identify a pre-existing NF-AT-binding factor that is present in hypotonic extracts of unstimulated T cells. On the basis of binding, reconstitution and cotransfection experiments, we propose that activation of NF-AT occurs in at least two stages: a CsA-sensitive stage involving modification and/or translocation of the pre-existing NF-AT complex, and a CsA-insensitive stage involving the addition of newly synthesized Fos or Fos/Jun proteins to the pre-existing complex.

We have characterized NF-AT-binding complexes from nuclear extracts of the untransformed murine helper T-cell clone Ar-5 (ref. 9). Using a murine NF-AT oligonucleotide (Fig. 1a) in electrophoretic mobility shift assays, two inducible DNA-protein complexes were detected in nuclear extracts from stimulated but not unstimulated Ar-5 cells (Fig. 1b, lanes 1 and 2). Both complexes were specific because they were competed by unlabelled NF-AT oligonucleotides derived from the murine (lane 3) and human (lane 4) IL-2 promoters. On the basis of methylation interference¹⁰, we designed the three mutant oligonucleotides (M1, M2 and M3) shown in Fig. 1a and tested them for direct binding (data not shown) and competition (Fig. 1b, lanes 5-7). The M1 oligonucleotide bound two inducible nuclear complexes and competed for binding as well as the unmutated oligonucleotide; the M2 oligonucleotide bound and competed less efficiently than M1, whereas the M3 oligonucleotide neither bound nor competed for binding. These results indicated that within the NF-AT oligonucleotide, the upper and lower complexes contacted the same core sequence, defined by mutant M3, and suggested that the adjacent sequence defined by mutant M2 was also required for optimal binding.

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Surprisingly, the upper complex was competed by oligonucleotides corresponding to the proximal AP-1 site of the IL-2 promoter¹¹ (Fig. 1b, compare lane 8 with 9) or to the metallothionein AP-1 site (data not shown); there was little effect on the intensity of the lower complex. Using the murine NF-AT probe with nuclear extracts made from several human and murine T-cell lines we found two complexes; again, the AP-1 oligonucleotides competed selectively for the upper complex. By contrast, a human NF-AT oligonucleotide¹ bound a single inducible nuclear complex which migrated to the same position as the murine upper complex, and was competed by AP-1 oligonucleotides.

We tested whether NF-AT-binding proteins were pre-existing in unactivated T cells (Fig. 1c). Because NF-AT-binding proteins are not detected in nuclei of unactivated T cells, we tested hypotonic extracts which contain mostly cytosolic proteins. Under conditions identical to those used for detecting nuclear NF-AT-binding proteins, a band migrating with mobility similar to the lower nuclear NF-AT complex was observed using the hypotonic extracts (lane 1). This complex had the same binding specificity as the nuclear NF-AT complexes, in that its binding was competed by the unlabelled NF-AT oligonucleotide (lane 2), and by the M1 and M2 but not M3 or IL-2κB oligonucleotides (lanes 3–6). Like the upper nuclear complex, the pre-existing NF-AT complex was competed by IL-2 AP-1 (lane 7) and metallothionein AP-1 (data not shown) oligonucleotides. These results demonstrate directly that resting T cells contain a pre-existing NF-AT binding factor, as suggested by Flanagan *et al.*⁸.

The ability of AP-1 oligonucleotides to compete for binding of the upper nuclear and the pre-existing NF-AT complexes led us to investigate whether either complex contained Fos or Jun (Fig. 2). An affinity-purified antibody against the Fos M pep-

tide¹² specifically altered the migration of only the upper NF-AT complex (Fig. 2, upper panel, lanes 1–4); similarly, an antiserum raised against a peptide (Pep1) common to the DNA-binding region of Jun proteins¹³ specifically blocked formation of the upper complex (lanes 5–8). The effects of both antibodies were specific, as equivalent concentrations of normal rabbit immunoglobulin did not diminish binding (lanes 9–12), and because the Fos and Jun antibodies did not influence the binding of inducible nuclear complexes from the same extracts to a labelled Sp1 oligonucleotide (data not shown). Two other affinity-purified Jun antibodies (Oncogene Sciences), raised against the Pep1 peptide and a second Jun peptide, also blocked formation of the upper nuclear complex without affecting the lower nuclear complex (data not shown). We suggest that the upper nuclear complex contains both Fos and Jun and represents the transcriptionally active NF-AT complex, whereas the lower nuclear complex may represent a core NF-AT-binding complex lacking Fos and Jun.

The pre-existing NF-AT complex reacted with one of the three Jun antisera tested (Fig. 2, lower panel, lanes 5–8) but not with the Fos antibody (lanes 1–4), suggesting that it expressed a Jun-related epitope or was associated with a Jun-related protein. Much lower concentrations of the Jun antiserum were required to inhibit formation of the pre-existing NF-AT complex compared with the nuclear NF-AT complex; this could be due to structural differences or to a higher concentration of proteins reactive with the Jun antiserum in nuclear extracts compared with cytosolic extracts. The intracellular location of the pre-existing NF-AT-binding factor remains undetermined: it might be cytosolic or might become extracted from the nucleus of resting cells under hypotonic conditions, as shown for the retinoblastoma¹⁴ and Ets-1 (ref. 15) proteins.

The function, and in most cases the induction, of NF-AT is inhibited by CsA and FK506, whereas induction of Fos/Jun messenger RNAs and proteins is not affected^{2-6,11,16}. However, a potential CsA-sensitive step in NF-AT activation may be some modification of Fos/Jun proteins required for their association with the NF-AT complex. To test this hypothesis, we used the Fos antibody to deplete the upper NF-AT-Fos-Jun complex from nuclear extracts of stimulated Ar-5 cells (Fig. 3a, lanes 1 and 2). Addition of these extracts, which contain only the lower NF-AT complex (lane 2), to nuclear extracts from CsA-treated, stimulated Ar-5 cells, which contained Fos and Jun proteins (data not shown) but not NF-AT (lane 3), resulted in appearance of a reconstituted upper complex (lane 4). This reconstituted complex was similar to the upper nuclear complex from stimulated T cells (lane 1), as judged by competition with intact or mutated NF-AT oligonucleotides (lanes 5-8) and reaction with the Fos antibody (lane 9). Reconstitution of the upper complex was not observed or was very weak if extracts from unstimulated Ar-5 cells, which contain no Fos and very little Jun¹¹, were used. These results indicate that AP-1 proteins are an inducible component of the upper nuclear NF-AT complex, and that their synthesis and modification are unaffected by CsA.

Addition of hypotonic extracts containing the pre-existing NF-AT-binding factor (Fig. 3b, lane 1) to nuclear extracts from cells stimulated in the presence of CsA (lane 3) resulted in the appearance of a complex migrating with similar mobility to the upper nuclear NF-AT complex (lane 4). Hypotonic extracts from unstimulated, CsA-treated T cells also contained the pre-existing complex (lane 2 and were able to reconstitute a similar 'upper' complex (lane 5), whereas nuclear extracts from unstimulated T cells did not reconstitute such a complex (data not shown). The reconstituted upper complex (lane 4) appeared similar to the upper nuclear complex from activated T cells (lane 11) with respect to its mobility, binding specificity (lanes 6-9), and reaction with the Fos antibody (lane 10). We postulate that this reconstituted upper complex contains one or more core NF-AT-binding subunits derived from the hypotonic extract of resting cells, as well as Fos/Jun proteins derived from the nuclear extract of CsA-treated, stimulated cells. But this reconstituted complex may not be structurally (or functionally) identical to the upper nuclear complex from activated T cells: the Jun

members present in the two complexes may differ, and the pre-existing NF-AT-binding subunit(s) may become modified upon activation.

The *in vivo* significance of the association between NF-AT and Fos/Jun was tested by cotransfecting Ar-5 cells with a *c-fos* expression plasmid¹⁷ and a chloramphenicol acetyltransferase (CAT) reporter plasmid containing a trimer of the region between -200 and -293 of the murine IL-2 promoter¹⁸ (pIL2CAT2/1-M, here called 3xNFAT/OCT-CAT). The 3xNFAT/OCT-CAT plasmid was inactive in unstimulated T cells, and cotransfection of the *fos* expression plasmid did not result in significant activation (Fig. 4, lanes 1 and 3). But when the cells were stimulated with anti-CD3 ϵ antibody, the induced level of CAT activity was higher in cells cotransfected with the *c-fos* plasmid than in cells cotransfected with a control plasmid lacking only the *c-fos*-coding sequences (compare lanes 2 and 4). The effect was most striking when the cells were minimally stimulated with low concentrations of anti-CD3 ϵ (compare lanes 5 and 6, 7 and 8). These experiments demonstrate a functional interaction between *c-fos* and NF-AT, consistent with the direct interaction observed in gel shift experiments. Moreover, they indicate that transcriptional activation by NF-AT must require some stimulation-dependent step in addition

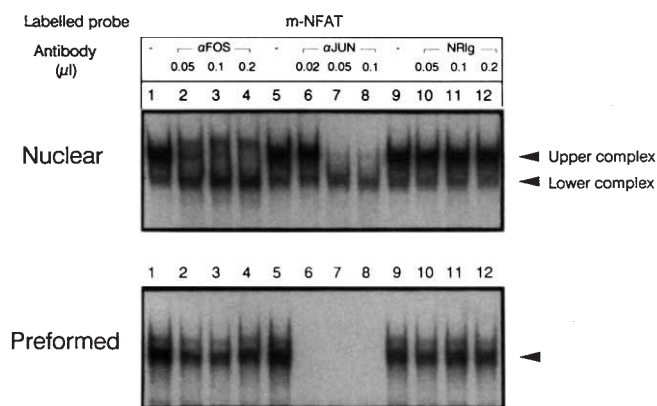


FIG. 2 The upper nuclear NF-AT complex contains Fos and Jun. Nuclear extracts from stimulated Ar-5 cells (upper panel) or hypotonic extracts of unstimulated cells (lower panel) were incubated with varying concentrations of an affinity-purified Fos antibody (1.25 mg ml⁻¹, lanes 2-4), an anti-Jun antiserum (lanes 6-8), or normal rabbit immunoglobulin (NRlg, 2 mg ml⁻¹, lanes 10-12) before addition of the labelled murine NF-AT probe. Lanes 1, 5 and 9 show binding assays done in the absence of antibody. Only the region of the gel containing the NF-AT complexes is shown. The 'supershift' observed with the Fos antibody is consistent with this antibody binding to the NF-AT-Fos-Jun complex and decreasing its mobility; by contrast, the Jun antiserum inhibits DNA binding.

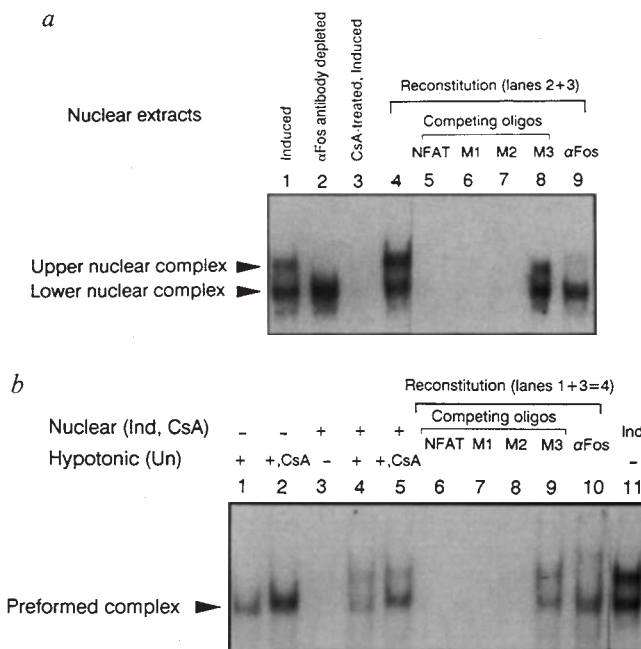


FIG. 3 Reconstitution of the upper nuclear NF-AT complex. *a*, Addition of nuclear proteins from T cells stimulated in the presence of CsA to Fos-depleted nuclear extracts. Nuclear extracts from stimulated Ar-5 cells (lane 1) were either left untreated or depleted of Fos and Fos-associated proteins by incubation with the Fos antibody and *Staphylococcus aureus* beads (lane 2). Nuclear extracts from cells stimulated in the presence of 1 μM cyclosporin A contained AP-1 (data not shown), but not NF-AT (CsA-treated, induced, lane 3); their addition to the Fos-depleted nuclear extracts restored the upper complex (lane 4). These reconstituted complexes were competed by unlabelled NF-AT, M1 and M2 but not M3 oligonucleotides (lanes 5-8), and shifted by the Fos antibody (lane 9). *b*, Addition of nuclear proteins from T cells stimulated in the presence of CsA to hypotonic extracts containing the pre-existing NF-AT complex. Hypotonic extracts from unstimulated (Un) or unstimulated, CsA-treated (Un, CsA) cells contained the pre-existing NF-AT complex (lanes 1 and 2), whereas nuclear extracts from cells stimulated in the presence of CsA (Ind, CsA) did not contain NF-AT binding activity (lane 3). When the extracts of lane 3 were mixed with those of lanes 1 and 2, an upper NF-AT-binding complex was restored (lanes 4,5). The reconstituted upper NF-AT complex had the same mobility as the upper complex present in nuclear extracts from stimulated (induced) Ar-5 cells (lane 11). It was competed by unlabelled NF-AT, M1 and M2 but not M3 oligonucleotides (lanes 6-9), and was shifted by the Fos antibody (lane 10).

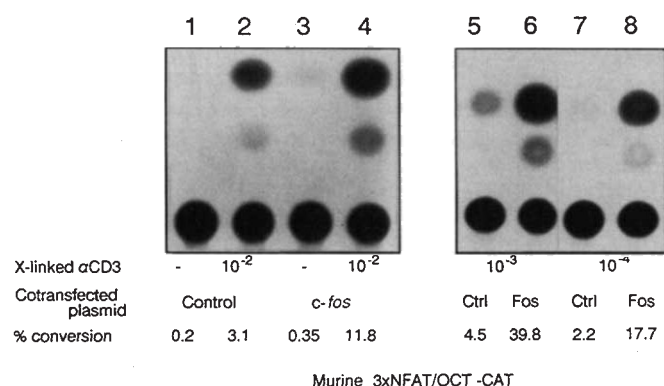


FIG. 4 Cotransfection of a *c-fos* expression plasmid augments the induction of the murine 3xNFAT/OCT-CAT plasmid in response to anti-CD3 ϵ stimulation in Ar-5 T cells. Ar-5 T cells were cotransfected as described^{11,26} with 2.5–5 μ g of the 3xNFAT/OCT-CAT plasmid and 2.5 μ g of either a human *c-fos* expression plasmid¹⁷ or a control plasmid lacking only the *c-fos* sequences. Transfected cells were activated the next day with the indicated concentrations of anti-CD3 ϵ and collected 24 h after activation. The per cent conversion of ¹⁴C-chloramphenicol to its acetylated forms was quantified using a Betagene Betascope. The left and right panels show two independent experiments.

to *c-fos* induction, because expression of *c-fos* in resting T cells does not activate NF-AT even though the pre-existing complex as well as all Jun proteins¹¹ are expressed.

These results are consistent with a model for NF-AT activation which requires two separate stimulation-dependent steps. One step is CsA-sensitive, and probably involves modification of the pre-existing NF-AT-binding factor and/or its translocation to the nucleus. The other step is insensitive to CsA, and involves the addition of newly synthesized Fos (and perhaps Jun) proteins to the pre-existing NF-AT-binding factor in the nucleus of stimulated T cells. We note that Fos (and Jun) proteins possess all the properties previously postulated for the newly synthesized subunit of NF-AT⁸: presence in nuclear rather than cytosolic extracts, ubiquitous expression in stimulated cells, and insensitivity to CsA and FK506.

Our results add to the increasing number of cases in which crosstalk between AP-1 and other transcription factors has been reported. Direct interactions between Fos/Jun proteins and steroid hormone receptors have been demonstrated^{19–22}, as have cooperative interactions between Ets proteins and AP-1 (ref. 23). In fact, the GGAA binding sequence²⁴ required for NF-AT binding is very similar to the core Ets binding sequence²⁴. Antibodies to an Ets-1-specific peptide and to a peptide shared between Ets-1 and Ets-2 (ref. 25) did not block binding in gel shift assays (data not shown); however, the NF-AT subunit may be related to a different member of the Ets family. Identification of the core NF-AT subunit(s), and elucidation of the mechanisms of NF-AT/AP-1 interaction, will be necessary to understand IL-2 gene induction and the mode of action of CsA and FK506. □

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Direct homeodomain-DNA interaction in the autoregulation of the *fushi tarazu* gene

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A MAJOR problem in the elucidation of the molecular mechanisms governing development is the distinction between direct and indirect regulatory interactions among developmental control genes^{1–5}. *In vivo* studies have indicated that the *Drosophila* segmentation gene *fushi tarazu* (*ftz*) directly or indirectly autoregulates its expression⁶. Here we describe a generally applicable experimental approach which establishes a direct *in vivo* interaction of the homeodomain protein *ftz* with the *ftz* cis-autoregulatory control region. *In vitro* studies have shown that the DNA-binding specificity of the *ftz* homeodomain can be changed by a single amino-acid substitution in the recognition helix (Gln 50 $\rightarrow$ Lys)⁷. Whereas wild-type *ftz* homeodomain binds preferentially to a CCATTA motif, the mutant homeodomain (*ftz*Q50K) recognizes a GGATTA motif. We now find that the *in vivo* activity of an *ftz* autoregulatory enhancer element is reduced by mutations of putative *ftz*-binding sites to GGATTA. This down-regulatory effect is specifically suppressed *in vivo* by the DNA-binding specificity mutant *ftz*Q50K. These results establish a direct positive autoregulatory feedback mechanism in the regulation of this homeobox gene.

Previous studies have identified a large autoregulatory control region in the *ftz* gene^{6,8}. This upstream region contains the 430-base-pair (bp) regulatory element AE (Fig. 1a) which directs *lacZ* reporter gene expression in seven *ftz*-like stripes in transgenic embryos (Fig. 1b). This expression is dependent on the *ftz* gene product itself, demonstrating that AE is a direct or indirect target for *ftz* autoregulation (Fig. 1c). We have previously identified one high- and five medium-affinity *ftz* *in vitro* binding sites⁸ in AE (termed A1–3, B, C and D in Fig. 1a). To test their *in vivo* role, binding sites were deleted in different combinations and *lacZ* reporter gene expression directed by mutant AEs was monitored in transgenic embryos (Fig. 2). Whereas single deletions that remove binding sites A1–3 (transgene AEΔA; Fig. 2a) or binding site D (AEΔD; Fig. 2d) have no or only a weak effect on enhancer activity, deletions of site B (AEΔB; Fig. 2b) or site C (AEΔC; Fig. 2c) clearly reduce reporter gene expression. The double deletion constructs removing sites A1–3 and D (AEΔAD; Fig. 2e) or A1–3 and C (AEΔAC; Fig. 2f) significantly reduce enhancer activity and the double deletion of C and D (AEΔCD; Fig. 2g) leads to a very great reduction of reporter gene expression. Triple deletions removing A, C and D (AEΔACD; Fig. 2h) reduce enhancer activity to

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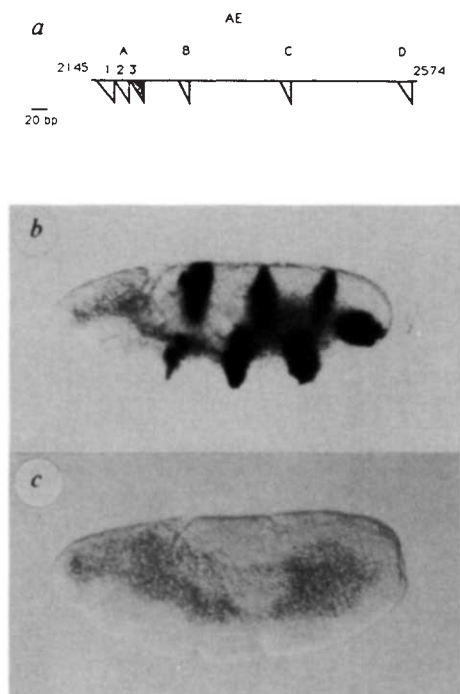


FIG. 1. Expression directed by the 430-bp *ftz* autoregulatory element AE. *a*, The AE region encompasses base pairs 2,145–2,574 (numbering according to ref. 25) in the upstream element²² and lies 3.5 kb upstream of the *ftz* transcriptional start site. AE contains most or all of the spatial information provided by the previously defined proximal autoregulatory element (ref. 8; A.F.S. and W.J.G., unpublished results) and includes all the sequences that are conserved between the proximal element of *D. melanogaster* and the proximal section of the *D. hydei* autoregulatory region²⁶. The AE contains five medium-affinity *ftz* *in vitro* binding sites (triangles A1, A2, B, C, D) and one high-affinity *ftz* *in vitro* binding site (triangle A3, ref. 8). It is not known if factors other than *ftz* protein bind to the regions encompassing the *ftz* *in vitro* binding sites. The AE and all subsequent derivatives were subcloned in forward orientation into the *hsp70-lacZ* reporter gene vector HZ50PL (ref. 6). This and all other fusion genes were introduced into the germ line of *Drosophila* by P-element-mediated transformation²⁷. Transformant embryos were stained for β -galactosidase activity by using the chromogenic substrate X-gal. Expression directed by AE was monitored in wild-type embryos (*b*) and embryos mutant for the *ftz* amorphic allele *ftz*^{9H34} (*c*). Anterior is to the left and dorsal is up in all figures.

METHODS. The region encompassing AE (bp 2,145–2,574) was amplified by polymerase chain reaction (PCR)²⁸ with the internal (sequences present in the *ftz* autoregulatory region) oligonucleotide primer ASPE23 (5'-CATTCATCTAGATTACGGGGTCATC-3') and an external (sequences present in the polylinker) oligonucleotide primer. The amplified fragment and all subsequent derivatives were gel-isolated, digested with *Xba*I and subcloned into the unique *Xba*I site of the reporter gene vector HZ50PL⁶. Orientation and sequences were verified by dideoxy sequencing. P-element-mediated transformation, establishment of balanced or homozygous transformant stocks, and detection of β -galactosidase activity was as described previously^{6,27}. For AE and all its derivatives, at least five independent lines were established and analysed. Transgenic embryos were stained for 48 h at 37 °C as described⁶.

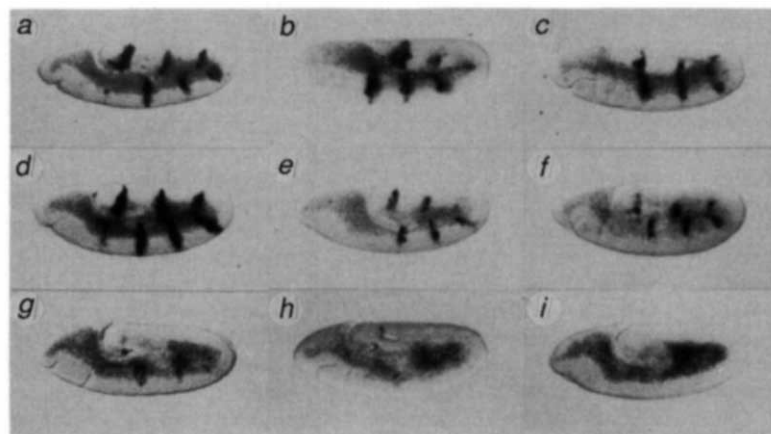
very low levels. Finally, removing all medium- and high-affinity binding sites (AE Δ ABCD; Fig. 2*i*) abolishes the activity of AE. These results indicate that the regions encompassing *ftz* *in vitro* binding sites are required for AE *in vivo* activity. All the sites seem to contribute to enhancer activity, although to different extents, and no single site is strictly required, suggesting that *ftz*-binding sites are combined in a partially redundant manner. This interpretation predicts that the *ftz*-binding sites are to some extent interchangeable. To test this model, binding site D was replaced with binding site A3 in the context of construct AE- Δ C. As expected for a partially redundant system, the resulting fusion gene AE-A3 is strongly expressed (Fig. 3*c*).

Although the deletion analysis provides evidence that *ftz* might directly interact with AE *in vivo*, *ftz* might activate another gene whose product then binds to AE and mediates autoregulation. Other homeodomain proteins expressed during early embryogenesis bind *in vitro* to sites very similar to the high-

affinity binding site A3 (refs 9–11). Therefore, it is conceivable that *ftz* activates another homeobox gene whose product binds to and acts through the putative *ftz*-binding sites in AE. A powerful genetic approach to identify directly interacting components is the isolation of compensatory second-site suppressor mutations¹². In this type of analysis a mutation affecting a given component is specifically suppressed by a mutation that causes a compensatory change in a second component with which the first physically interacts (see for example, refs 13 and 14). To find out if *ftz* protein binds directly to AE *in vivo*, we made use of results from structural and genetic studies on the interaction of homeodomains with their DNA binding sites^{7,15–20}. These investigations have shown that amino acid 50 of the homeodomain (amino acid 9 in the recognition helix) is an important determinant of DNA-binding specificity. This residue contacts base pairs 5' of the ATTA core, which is common to many homeodomain binding sites. In the case of *ftz*, wild-type *ftz*

FIG. 2. Deletion analysis of the *ftz* *in vitro* binding sites in AE. AE fragments missing different *ftz* *in vitro* binding sites were constructed and expression of fusion genes was monitored in transgenic embryos. Expression of fusion genes: *a*, AE- Δ A (which contains sequences 2,224–2,574); *b*, AE- Δ B (2,145–2,265 and 2,276–2,574); *c*, AE- Δ C (2,145–2,393 and 2,418–2,574); *d*, AE- Δ D (2,145–2,542); *e*, AE- Δ AD (2,224–2,542); *f*, AE- Δ AC (2,224–2,393 and 2,418–2,574); *g*, AE- Δ CD (2,145–2,393 and 2,418–2,542); *h*, AE- Δ ACD (2,224–2,393 and 2,418–2,542); and *i*, AE- Δ ABCD (2,224–2,265, 2,276–2,393 and 2,418–2,542). No expression of AE- Δ ABCD is detectable in four out of six transgenic lines.

METHODS. All deletion derivatives of AE were generated by PCR using previously described strategies²⁹. To create internal deletions, two inner oligonucleotide primers introducing deletions were used in two separate PCR reactions extending in opposite directions from the site of the deletion. The gel-purified fragments of the first sets of reactions were annealed and used for a second round of PCR with outer primers. (For detailed protocols and sequences of oligonucleotides, see Supplementary Information.)



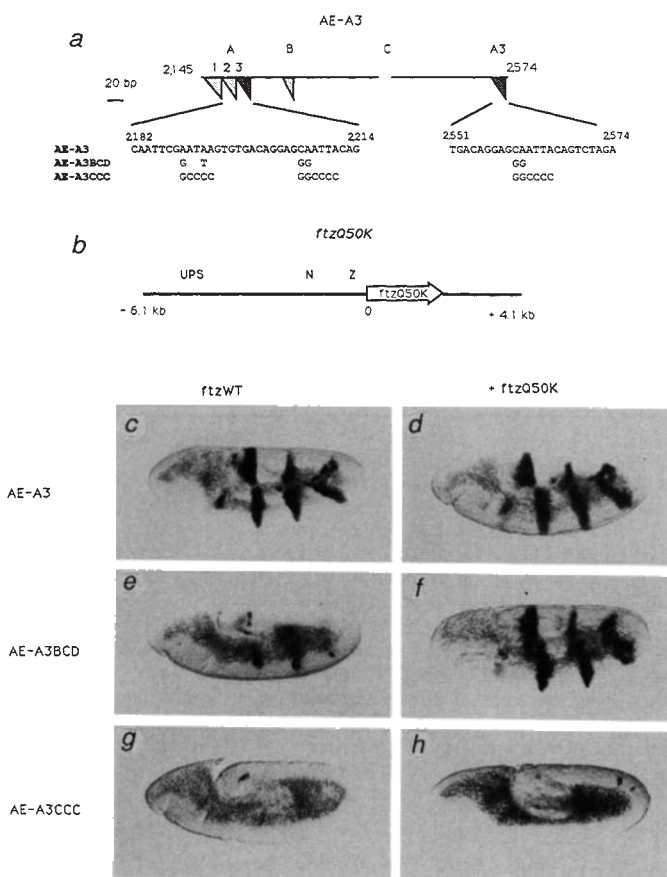


FIG. 3 A DNA-binding specificity mutant of *ftz* suppresses the down-regulatory effects caused by corresponding mutations of *ftz* *in vitro* binding sites in AE. **a**, Structure of the AE-A3 element and its derivatives. *Ftz* *in vitro* binding site D in AE- Δ C was replaced with binding site A3. *Ftz* *in vitro* binding sites A2 and A3 in AE-A3 were mutated to *bcd*-binding sites giving rise to AE-A3BCD. The ATTA core of the *bcd*-binding sites was mutated further to CCCC, giving rise to AE-A3CCC. AE-A3BCD and AE-A3CCC also miss *ftz* *in vitro* binding site A1. Mobility shift assays reveal that the *ftz*WT

homeodomain binds *in vitro* with high affinity to a CCATTA (refs 7, 11) or CAATTA motif (ref. 8), but only weakly to a GGATTA motif^{7,18}. This motif is present in binding sites for the homeodomain protein bicoid (*bcd*)²¹. Converting amino acid 50 in the *ftz* homeodomain from Gln to Lys (as found in *bcd*) enables the mutant *ftz* homeodomain (*ftz*Q50K) to bind with high affinity to the *bcd*-binding site⁷. To determine if *ftz* protein interacts directly with the *ftz* *cis*-control region *in vivo*, we applied these *in vitro* observations, on the basis of the following logic. If wild-type *ftz* (*ftz*WT) directly recognizes AE *in vivo*, an AE whose putative *ftz*-binding sites were mutated to *bcd*-binding sites would have a reduced affinity for *ftz*WT and would have reduced enhancer activity *in vivo*. Introduction of a transgene encoding the *ftz* DNA-binding specificity mutant *ftz*Q50K into this genetic background could have two possible consequences on the expression of the mutant AE. In the case of a direct autoregulation, *ftz*Q50K would be able to recognize the mutant AE and act as a dominant, allele-specific, second-site suppressor restoring enhancer activity. Alternatively, in the case of an indirect autoregulation, *ftz*WT would act through another gene whose product then in turn binds to AE. In this case no suppression would be expected on introduction of *ftz*Q50K. Specific suppression could only be achieved by changing the DNA-binding specificity of the (unknown) gene product mediating *ftz* autoregulation through the putative *ftz* binding sites.

Ftz binding sites A2 and A3 in AE-A3 were mutated to *bcd* consensus binding sites (AE-A3BCD, Fig. 3a). As a negative

homeodomain binds with high affinity to the A3-binding site and with a roughly 10-fold and 100-fold reduced affinity to site A3BCD and site A3CCC, respectively. By contrast, *ftz*Q50K homeodomain and *bcd* homeodomain bind with high affinity to A3BCD and with at least 10 times lower affinity to A3 and A3CCC (data not shown). **b**, Structure of the mutant *ftz* gene *ftz*Q50K that carries a single base pair change converting amino acid 50 in the homeodomain from Gln to Lys. This transgene carries all the necessary regulatory elements to direct the expression of *ftz*Q50K in the domains of *ftz*WT. UPS, upstream element; N, neurogenic element; Z, zebra element²². Expression directed by the different AE-A3 derivatives was monitored in wild-type embryos (**c**, **e**, **g**) or in wild-type embryos containing in addition one copy of the *ftz*Q50K transgene (**d**, **f**, **h**). **c**, **d**, Expression directed by AE-A3; **e**, **f**, expression directed by AE-A3BCD; **g**, **h**, expression directed by AE-A3CCC. Although there are three high-affinity bicoid binding sites in fusion gene AE-A3BCD, no expression is detectable in the anterior region of transgenic embryos, where *bcd* protein is present at high levels during early embryogenesis (**e**). This agrees with a previous study that reported that a regulatory element that contains five *bcd*-binding sites failed to direct expression of the *hsp 70-lacZ* reporter gene used in our study³⁰. When joined to the basal promoter of the *Krüppel* gene, the same element was able to direct gene expression in the early embryo. These findings suggest that the ability of *bcd*-binding sites to act in an enhancer element is strongly dependent on the reporter gene construct used^{21,30}. Expression of AE-A3CCC is comparable to the deletion construct removing sites A, C and D (AE Δ ACD in Fig. 2h), confirming the results of the binding-site deletion analysis. The presented data provides direct *in vivo* evidence for the binding of *ftz* protein to region A of AE. It is possible that factors other than *ftz* bind to AE, including the regions encompassing the *ftz*-binding sites.

METHODS. To generate the different AE-A3 derivatives, we used PCR as described (Fig. 1 legend) and fusion gene AE Δ C as a template. In AE-A3 the underlined sequences encompassing region D (2,551 TGAAGATTCTTCAATTATCTAGA 2,574) are replaced with the underlined sequences encompassing region A3 (2,551 TGACAGGAGCAATTACAGTCTAGA 2,574). The mutant *ftz* gene *ftz*Q50K was obtained by site-directed mutagenesis (Amersham's oligonucleotide-directed *in vitro* mutagenesis system version 2). The oligonucleotide Q50KASmrfl (5'-CATGCGTCGGTTTTGAACAGATCTTG-3') was used to mutate the codon CAA encoding Gln at position 50 of the *ftz* homeodomain to codon AAA encoding Lys. With the exception of some polylinker sequences and the Q50K mutation, the *ftz*Q50K transformation vector (pC20ftzQ50K) is identical to the transformation vector prf20 (corresponding to the P element P(*ry*,*ftz*G)) which complements *ftz* mutations²². Detailed cloning protocols and oligonucleotide sequences are available (see Supplementary Information). Four independent transformant lines were established for *ftz*Q50K. Embryos were collected and stained from crosses of flies carrying the different AE-A3 derivatives with flies carrying the *ftz*Q50K transgene.

control, the ATTA core of these sites was further mutated to CCCC (AE-A3CCC, Fig. 3a). Expression of AE-A3BCD is reduced (Fig. 3e) as compared with the wild-type construct (Fig. 3c) but not as severely as expression of AE-A3CCC (Fig. 3g). This suggests that *ftz*WT binds and activates weakly through the *bcd*-binding sites in AE-A3BCD.

To test for a direct autoregulatory interaction, a single base pair in the 10-kilobase (kb) *ftz* gene²² was mutated *in vitro* to convert amino acid 50 in the homeodomain from Gln to Lys (transgene *ftz*Q50K, Fig. 3b). Transgenic fly lines were established for *ftz*Q50K, crossed to lines carrying the different AE-A3 derivatives and reporter gene expression was analysed in their progeny. As expected for the case of a direct autoregulatory interaction, the presence of *ftz*Q50K restores enhancer activity of the AE-containing *bcd*-binding sites (Fig. 3f). This suppression is seen neither with an additional copy of *ftz*WT (data not shown) nor if the *bcd*-binding sites are mutated (Fig. 3h). These *in vivo* results, together with previous genetic and *in vitro* binding studies⁶⁻⁸, provide compelling evidence that part of the molecular basis of the self-enhancement of *ftz* gene expression is a direct positive autoregulatory feedback loop in which the homeodomain protein *ftz* binds to and enhances transcription through the *ftz* *cis*-regulatory region. Note that the finding of direct autoregulation does not exclude the possibility that there might also be an indirect effect in *ftz* autoregulation.

Our *in vivo* data confirm two important observations previously made *in vitro* and in cell culture systems. First, we find

that *ftz* acts as a DNA-binding transcriptional activator during *Drosophila* embryogenesis^{4,5,23,24}. Secondly, our results verify that amino-acid residue 9 in the homeodomain recognition helix is a major determinant of the DNA-binding specificity of homeodomain proteins and contacts bases just 5' of the ATTA core motif^{7,15-20}.

The demonstration of direct *ftz* autoregulation was made possible by combining the classic genetic concept of allele-specific, second-site suppressor mutations with the *in vitro* design of DNA-binding specificity mutants. This class of

mutants has so far been used to define specific amino acid-base pair interactions in protein-DNA complexes or to identify rules that assign members of a gene family to subfamilies with different target site specificities^{7,14-17}. We have now reported the novel use of a DNA-binding specificity mutant as a tool to establish a direct interaction of a transcription factor and a DNA target site in a multicellular organism. This *in vivo* approach can be applied to any system where transgenic organisms can be generated and should help the analysis of the complex regulatory networks governing development. □

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SUPPLEMENTARY INFORMATION. Requests should be addressed to the London editorial office of *Nature*.

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***In vitro* guide RNA/mRNA chimaera formation in *Trypanosoma brucei* RNA editing**

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THE post-transcriptional processing of various mitochondrial transcripts in kinetoplasts, kRNA editing, adds and removes uridines, producing mature messenger RNAs^{1,2}. This editing seems to be directed by 'guide' RNAs (gRNAs) which are complementary to portions of the mature message³. The editing mechanism has been proposed to entail transesterification^{4,5}. Detection of chimaeric gRNA-mRNA molecules, intermediates predicted by transesterification, support this model⁴. We report here the *in vitro* formation of such chimaeras where endogenous gRNAs are covalently linked to added synthetic mRNA. Addition of gel-purified gRNAs to the standard reaction mix increases chimaera formation. This increase is not observed when the gRNA 3'-hydroxyl group is chemically modified, identifying this terminal hydroxyl as the reactive group. These results provide the first experimental evidence for an *in vitro* RNA editing event and support the involvement of transesterification as a chemical mechanism.

All but the terminal regions of ATPase 6 (A6) mRNA are extensively edited in *Trypanosoma brucei*⁶ and we have identified several gRNAs that could direct this editing. This includes gA6-14 which is complementary to the furthest 3' edited sites⁶. The 5' end of gA6-14 is complementary to 12 nucleotides (nt) at the 3' end of the A6 message which are not edited in the mature mRNA (Fig. 1a). This complementary region can form an 'anchor' duplex which has been proposed to initiate the editing process⁵. We constructed a clone, 3'A6UK, such that 100 nt of pre-edited A6 mRNA with 72 nt of vector sequence

at its 3' end is produced on *in vitro* transcription (Fig. 1b). These transcripts were incubated in mitochondrial extracts to determine whether endogenous gRNAs would transesterify *in vitro*. Complementary DNAs were then made using reverse transcriptase and BS-KS, an oligonucleotide primer specific for the vector sequence. The resultant cDNAs were amplified by polymerase chain reaction (PCR) using BS-KS and gA6-14c, an oligonucleotide specific for the gA6-14 gRNA.

Chimaeric molecules formed between the added mRNA and gRNAs should yield a PCR product of 116 base pairs (bp) or larger, depending on the site of transesterification. PCR products between 120 and 180 bp were visible when stained with ethidium bromide after two rounds of 30 cycles of PCR amplification. Cloning and sequencing of the PCR products showed that endogenous gA6-14 gRNAs were covalently linked to the synthetic mRNA at a variety of editing sites (Fig. 2). The 35 chimaeric gRNA-mRNA clones yielded 17 different sequences, all with the gRNAs linked in the editing domain of the A6 mRNA. Most of the cDNAs have the gRNA linked in editing sites 1-16, which are specified by gA6-14. Three cDNAs (A6CIV31, 21 and 45) have the gRNA linked at sites 5' to the region that complements gA6-14 indicating that this gRNA can edit this region⁷. The mRNA downstream of the gRNA is unedited except in A6CIV14 which lacks one of the two uridines that are deleted in mature mRNA. This single case may represent complete *in vitro* editing at this site. Control PCR amplifications using an oligonucleotide specific for gA6-48 (refs 6, 7), an A6 gRNA that cannot form an anchor duplex with the input RNA substrate, did not generate any chimaeric product.

The 3' region of the gRNA portion of the chimaeric molecules varies by as much as 15 nucleotides. The longest gRNA sequence in the chimaeras extends 3 nucleotides beyond the region of perfect duplex between the gRNA and mRNA. Most of the gRNA sequences were 8-11 nucleotides shorter than the gRNA sequence in Fig. 1. This heterogeneity in the 3' region of the gRNA is also observed in chimaeras derived from *in vivo* RNA⁸. Only three of the 17 different chimaeric molecules contain non-encoded uridines at the gRNA-mRNA junction. In the transesterification model of editing, uridines are provided by

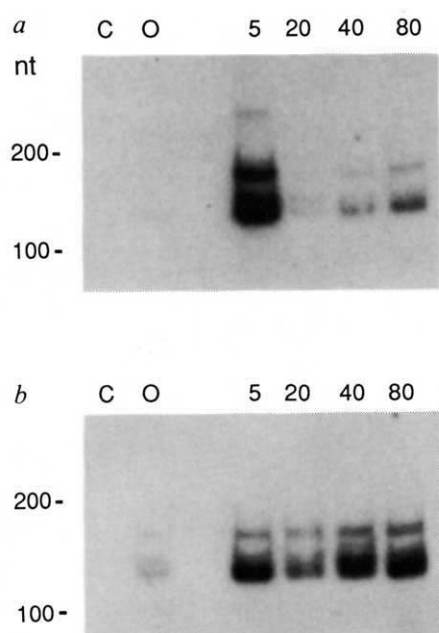


FIG. 3 Southern blot analysis of a time course for chimaera formation. The 3'A6UK was incubated for 0, 5, 20, 40 and 80 min at 27 °C (a) or 37 °C (b) and processed as described in Fig. 2 legend. Half of the product from the first round of PCR was run on a 6% polyacrylamide-TBE (89 mM Tris-borate, pH 8.3, 1 mM EDTA) gel and blotted onto Nytran for 30 min at 0.5 mA cm⁻² using a TE77 SemiPhor electroblotter. Blots were hybridized at 55 °C for 12 h with ³²P-end-labelled A64-7 oligonucleotide and washed at 60 °C as previously described¹³. C, No lysate, control lane. For the zero time point, SDS/TE stop buffer was added to the reaction mix before addition of the RNA substrate. The chimaeric nature of the hybridizing signal was confirmed by gel purification of the bands, and cloning and sequencing as described for Fig. 2. The broad lower band corresponds to a mixture of chimaeras with the gRNA linkage at sites 1–10. The distinct upper band corresponds to the A6CIV21 chimaera. Calibration (in nucleotides) shown on the left.

that chimaera formation is limited by gRNA abundance in these experiments. This enhancement is not observed when the added gRNAs are pre-treated with sodium periodate or 3' end-labelled with 3',5'-cytidine bisphosphate (Fig. 4b–d). Sodium periodate oxidizes the vicinal 2',3'-hydroxyl groups of the terminal ribose, whereas 3' end labelling creates a terminal phosphate^{11,12}. Both treatments modify the 3' ends, thereby making them unavailable for transesterification or ligation. Chimaera formation was not inhibited by addition of sodium periodate-treated nonspecific RNAs, indicating that inhibition is specific to gRNA 3' end-modification and not residual sodium periodate. These results suggest that the formation of a covalent linkage between gRNAs and mRNAs requires the presence of an active hydroxyl group at the 3' terminus of the gRNA.

Here we have described the development of an *in vitro* system that covalently links gRNAs to mRNAs in a reaction predicted for RNA editing. In this system, the initial linkage can occur at

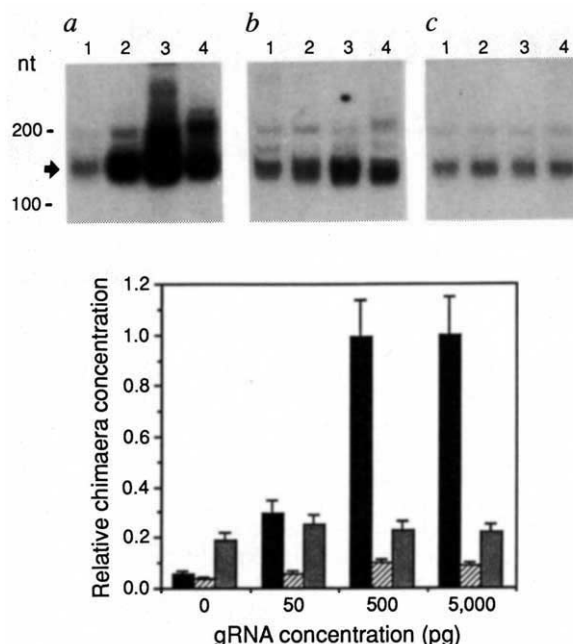


FIG. 4 Analysis of chimaera formation on addition of gRNAs and 3' end-modified gRNAs. Isolated untreated gRNAs (a), sodium periodate-treated gRNAs (b) and 3' end-labelled [5'-³²P]pCp gRNAs (c) were incubated in mitochondrial extract with 3'A6UK mRNA for 10 min at 27 °C as described for Fig. 2. Lanes 1–4, 0, 50 pg, 500 pg and 5,000 pg of added isolated gRNAs, respectively. Amplification product was run on 6% polyacrylamide gels and blotted and probed with A64-7 as described for Fig. 3. d, Comparison of relative chimaera formation after incubation with untreated (solid bars), sodium periodate treated (hatched bars) or 3' end-labelled [5'-³²P]pCp (dotted bars) gRNAs. The most abundant chimaeric band was used in the quantification (arrow in a, b, c). Relative errors as indicated by the error bars were determined to be $\pm 15\%$. Biomarker LOW (BioVentures) size markers are indicated in nucleotides.

METHODS. *T. brucei* procyclic gRNAs were gel-purified as previously described (H.U.G. *et al.*, manuscript in preparation). The free hydroxyl groups of the gRNAs 3'-terminal uridine nucleotide were oxidized with a 100-fold molar excess of sodium periodate in 0.1 M sodium acetate, pH 4.5 (ref. 11). The reaction was done in the dark at 4 °C for 2 h. Excess sodium periodate was inactivated by adding sucrose at 700 $\times$ molar excess over NaIO₄ and the RNA was precipitated with ethanol. The 3' end-labelling was done with T4 RNA ligase (BRL) and [5'-³²P]3',5'-cytidine bisphosphate (NEN) according to ref. 12. Non-saturated autoradiograms were scanned for quantification using a BioRad model 620 densitometer.

many different sites in the editing domain⁷ and does not require a terminal uridine residue. But the generation of the covalent linkage between gRNA and mRNA does require a 3'-hydroxyl group at the terminal end of the gRNA. This indicates that chimaeric molecules are editing intermediates and supports the kRNA editing model in which uridine deletions and additions are accomplished by a transesterification mechanism analogous to that of RNA splicing^{4,5}. □

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Continuous beds: high-resolving, cost-effective chromatographic matrices

Stellan Hjertén, Yi-Ming Li, Jia-Li Liao, Jamil Mohammad, Ken'ichi Nakazato and Göran Pettersson

The need for new types of beds (continuous beds), their unique preparation and attractive properties are discussed.

ACCORDING to classical chromatographic theory, a plot of the width of a zone (or more correctly the plate height) versus the flow rate exhibits a minimum. For macromolecules this minimum, which corresponds to a maximum in resolution, occurs at such low flow rates that the run times become unacceptably long. Most chromatographic experiments are therefore performed at flow rates that do not provide optimum resolution. For this reason, we posed the following question. Is it possible to design a bed such that the width (the plate height) of a zone is independent of the flow rate or even decreases with an increase in flow rate? The answer is 'yes'.

Design of the bed

The first test experiments were done with nonporous agarose beads. They were prepared by a suspension-gelation procedure and then modified to obtain beds with this highly desirable relationship between zone width and flow rate¹⁻¹⁰. The technique to prepare the unmodified beads, the principle of which is used routinely for manufacturing beads of various bed materials, is very time-consuming and expensive¹¹. Therefore, we are developing a new and simple method for the synthesis of beds with the same advantageous chromatographic properties as the above agarose columns¹²⁻¹⁴ (see Fig. 1). An aqueous solution of proper monomers (for instance,

derivatives of acrylamide), a catalyst and salt is poured into the chromatographic tube. In the presence of salt, the polymer chains formed aggregate into large bundles by hydrophobic interaction, creating between the bundles voids (irregularly shaped channels) large enough to permit a high hydrodynamic flow. Following polymerization, the bed is compressed by connecting it to an HPLC pump adjusted to a flow rate equal to or higher than that to be used in a subsequent run. The bed can be compressed further with the aid of the upper movable plunger. The bed obtained, which we call a 'continuous bed', can be regarded as a rod or plug permeated by 'channels' in which the eluent can pass upon application of pressure. The walls of the continuous beds thus correspond to the agarose beads and the channels to the void between the beads.

The greatest problem in the design of the continuous gels was to find experimental conditions for the polymerization such that the channels, upon compression of the bed, did not become too small to allow passage of buffer through the column. Therefore, the walls in the continuous beds must not be excessively soft and deformable or so rigid that they become fragile and break down upon application of pressure. Beds designed properly will stand a pressure of 400 bar, although most experiments require less than 50 bar, even at high flow rates (see Fig. 2). Even peristaltic pumps can be used if the requirement of very short run times is not essential.

Characteristics of the bed

Typical features of the continuous beds are that the walls of the channels (1) are impermeable to peptides and proteins, eliminating diffusion of the solutes into and out of the wall; (2) have a rough surface, affording the possibility to re-

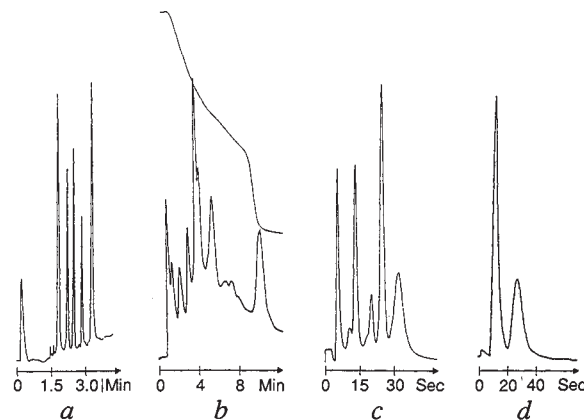


FIG. 2 High-performance liquid chromatography on continuous gels. a, Reversed-phase chromatography of model proteins. Bed dimensions, 6-mm (i.d.) $\times$ 35 mm; flow rate, 5 ml min⁻¹; pressure, 60 bar. b, Chromatofocusing of a yeast extract. Bed dimensions, 6-mm (i.d.) $\times$ 27 mm; flow rate, 0.5 ml min⁻¹; pressure, 36 bar. c, Micro cation-exchange chromatography of model proteins. Bed dimensions, 0.3-mm (i.d.) $\times$ 192 mm; flow rate, 0.12 ml min⁻¹; pressure, 24 bar. d, Chiral-recognition chromatography of racemic proctolol on a continuous bed derivatized with cellulase as an enantioselective agent. Bed dimensions, 6-mm (i.d.) $\times$ 40 mm; flow rate, 7 ml min⁻¹; pressure, 100 bar.

place laminar flow with non-laminar flow with attendant enhancement of the velocity of transport of the solutes between the walls (in laminar flow the transport takes place only by diffusion, which is a slow process); (3) are deformable to permit compression of the bed, thereby decreasing the diameter of the channels, that is, the distance between the walls (the effect is similar to that produced by using beads of extremely small diameters⁹); (4) show negligible nonspecific interaction, favoring a rapid adsorption-desorption of the solutes.

It is outside the scope of this article to discuss further the above hypothesis regarding the separation mechanism. We will stress that we have no experimental evidence to suggest that non-laminar flow is created in the beds or that the lift force discussed in ref. 1 is operative. It is, however, interesting to note that a literature search showed that the existence of such flow in conventional chromatographic columns was postulated many years ago to explain why the plate height decreased upon an increase in flow rate¹⁵⁻¹⁷ (these observations were, however, not taken advantage of and utilized for improvement of the resolution).

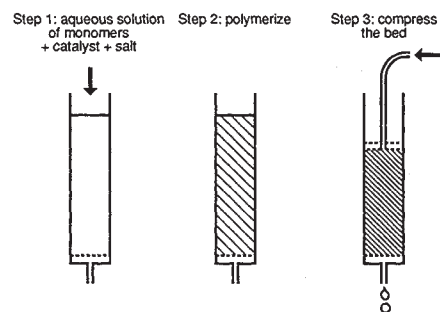


FIG. 1 The principle for the preparation of a continuous bed. The chromatographic tube is filled with an aqueous solution of appropriate monomers, including that of the ligand, catalyst and salt (step 1). Following polymerization (step 2), the bed is compressed (step 3). The polymerization proceeds under conditions such that irregular channels form in the bed through which the eluent can pass.

Simple derivatization

Conventional chromatographic supports are often synthesized in two steps: firstly, the beads are prepared and then they are derivatized for immobilization of the desired ligand. The continuous beds have the advantage that the immobilization takes place at the same time as the matrix is synthesized. For instance, for the preparation of a cation-exchanger, acrylic acid is included in an aqueous monomer solution of acrylamide derivatives supplemented with a catalyst and salt¹².

A similar approach has been used for the synthesis of beds for anion-exchange chromatography¹³, hydrophobic-interaction chromatography¹³, affinity chromatography (J. M. and S. H., manuscript in preparation), chromatofocusing¹⁴, reversed-phase chromatography¹⁸ and chiral-recognition chromatography¹⁸. As prepared now, the beds are stable in the pH range 1–11 for at least three weeks — the test period¹⁴. As the surface of the nonporous walls of the irregular channels is very rough, the protein capacity is relatively high. For an anion-exchanger, it was about 12 milligrams of haemoglobin per millilitre of packed bed¹⁴.

As the gel bed is not made up of free, discrete particles, but rather of particles connected to each other (diameter about 0.5 µm as measured from a scanning electron micrograph)¹³, it is not necessary to use a supporting frit at the bottom of a gel, provided that the gel bed is covalently linked to the wall of the chromatographic tube and that its diameter is as small as in the microchromatography experiment presented in Fig. 2c.

Future perspectives

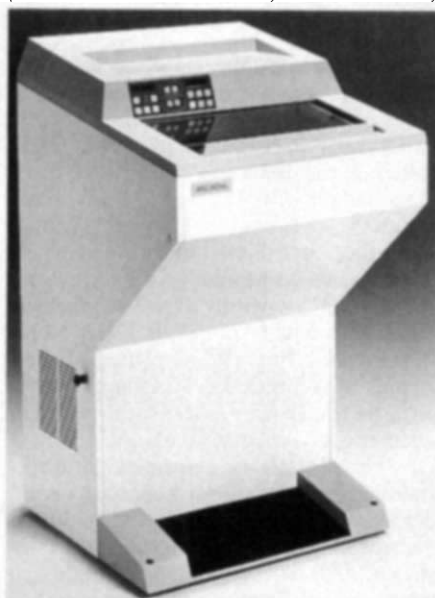
Research into the design of continuous beds began only a few years ago. Consequently, we are in only the early stages of developing new approaches to preparing chromatographic columns. The technique is still being modified to obtain beds with improved chemical and mechanical properties. The chromatograms in Fig. 2 indicate that the continuous gels, even given the present 'state of the art', afford high resolution in combination with short analysis times, which is a characteristic feature of HPLC. As the preparation of continuous beds is cost-effective, they also have potential in the field of large-scale chromatography □

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Analytica '92

Over 900 exhibitors will be represented at Analytica '92, the 13th International Trade Fair for Biochemical and Instrumental Analysis to be held next week in Munich, Germany.

TEMPERATURES as low as -35°C and an integrated freezing stage for up to six specimens are features of the new HM 505 series of **cryostats** from Carl Zeiss (Reader Service No. 101). These sealed,



Cryostats for routine clinical and research use.

frost-free, open-top instruments with illuminated stainless-steel freezing chambers are equipped with a heated sliding window that is designed to eliminate condensation and provide a clear view of the chamber. The new cryostats feature a high-performance monoblock microtome for stability, which provides a feed range of up to 28 mm. Zeiss says the cross-roller bearings require no maintenance and ensure smooth, vertical specimen movement. Interchangeable knife carriers for standard knives or disposable blades accommodate specimens up to 70×50 mm.

Other features of the cryostats include precise x , y and z orientation of the specimen, a lockable hand wheel, anti-roll guides for knife carriers and a warning bell that sounds when the user approaches the feed range limit. Designed with the user in mind, the instruments feature a heated arm rest and a spacious cooling chamber that provides easy access and ample storage space. HM 505 cryostats are supplied in both manual and motorized models. The motorized cryostat offers blade retraction and 15 trimming modes. Section and trimming thickness settings can be set from 1 to 500 µm.

To complement its range of polymer molecular weight standards, Polymer Laboratories has introduced a range of **PL-NanoCal NIST-traceable particle size standards** (Reader Service No. 102). Polymer Laboratories says the particle size standards are manufactured under stringent quality-control conditions and are highly characterized by analytical criteria, facilitating traceability to the particle size standards of the National Institute of Standards and Technology. Stated applications include the standardization and calibration of particle sizing and counting equipment, as filter challenge markers and in surface analysis, *in vivo* immunology and circulation and *in vitro* cell uptake studies. The particle size standards are available in nominal mean diameters at 50-nm increments between 100 and 1,000 nm. Three calibration kits of 9×5 -ml samples in the size range 100–500 nm and 500–1,000 nm (50-nm increments) and 100–1,000 nm (100-nm increments) are available. Individual PL-NanoCal particle size standards also are available in quantities of 5 and 15 ml at two per cent w/w solids.

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DNA details

With its new Gene Navigator system, Pharmacia hopes to make **pulsed-field gel electrophoresis** simple and accurate enough for it to be used as a standard research tool for mapping and DNA purification (*Reader Service No. 103*). The system combines the practical benefits of the hexagonal electrode technique with specially designed instrumentation, and is intended to make the separation and purification of large plasmids, YAC mapping and mapping of chromosomes easy and reproducible. Pharmacia says that resolution, which is good for separations from 20 kilobases to one megabase, also extends up to 12 million base pairs. The gel support tray and gel casting frame facilitate gel handling and loading of the samples, and internal buffer circulation and cooling coils are intended to provide an even temperature distribution, resulting in straight lanes and high resolution.

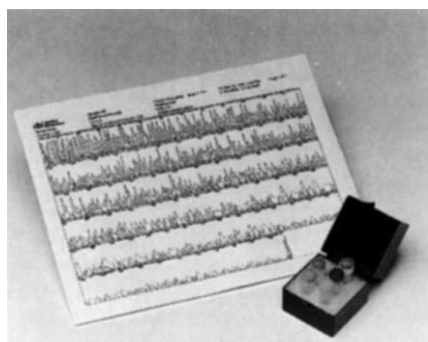
Bio-Rad has introduced the GS Gene Reader **automated film reader**, which is designed to read automatically DNA sequencing autoradiograms with high accuracy by using signal processing algorithms and a high-resolution, custom-designed film scanner (*Reader Service No. 104*). With this set up, which comprises an automated film scanner and an automated base calling system, a linear array CCD



Bio-Rad's automatic sequence reader.

digitizes the image and pattern recognition software identifies lane sets, corrects for artifacts and assigns bases. According to Bio-Rad, the accuracy of the GS Gene Reader is sufficient to allow direct entry of unedited base calls into a sequence assembly software package. To facilitate final editing, the film image is displayed on the computer screen in an enhanced and enlarged format alongside the assigned sequence. Difficult regions are flagged, so only certain areas may need to be confirmed.

PRISM is the new ready reaction kit from Applied Biosystems for **dye-primer chemistry** on the company's 373A DNA sequencer (*Reader Service No. 105*). As the reaction brews are premixed, the number of steps required for extension reactions with PRISM is much lower than



PRISM — ABI's kit for dye-primer sequencing.

is required with the standard kit. ABI says, fewer manual steps should result in less error and more consistent data from experiment to experiment. Each lot is tested on the 373A to verify that it meets the standard of 450 bases at 98 per cent accuracy on a double-stranded plasmid. The ready reaction chemistry uses a means of template amplification called 'cycle sequencing', whereby DNA template is combined with AmpliTaq DNA polymerase and linearly amplified during rounds of controlled heating and cooling on a thermal cycler. ABI says cycle sequencing offers several advantages: less DNA template is required; double-stranded templates are automatically heat-denatured; requirements for the quality of the template are less stringent; large constructs such as cosmids and lambda can be sequenced directly; and single-stranded and double-stranded template can be sequenced with one protocol. ABI says the method also has proven effective with PCR-generated templates.

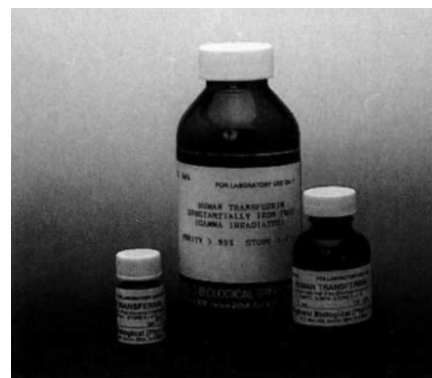
For the reproducible pre-exposure of autoradiography film, Amersham recommends use of Sensitize, a flashgun designed specifically for **hypersensitizing autoradiography film** (*Reader Service No. 106*). While the requirement for pre-exposure of film to an instantaneous flash of light in order to obtain quantitative accuracy and optimal sensitivity in fluorography (and when using intensifying screens) is well known, its importance and effectiveness, Amersham says, is often underestimated. Researchers who, until now, have relied on a standard photographic flash unit modified in-house with red tape, now have an alternative. Sensitize comprises a pre-flash unit with optimized filter, and protocols for calibration and routine use. Optimized for use with Hyperfilm-M and Hyperfilm-ECL in conjunction with Hyperscreens or the fluorographic reagent, Amplify, Sensitize is completely sealed to guard against white light leakage, which can be a problem with conventional flashguns.

Bind-Aid amplification enhancer from United States Biochemical, a formulation of *Escherichia coli* single-strand

DNA binding protein, is designed to improve the specificity and yield of the polymerase chain reaction (PCR) (*Reader Service No. 107*). It functions by accelerating the annealing of complementary oligonucleotide primers to the target DNA while binding to and inhibiting the extension of mismatched oligonucleotide primers. USB says Bind-Aid is most effective when used in conjunction with the 'hot start' technique and with an otherwise optimal PCR reaction mixture. In general, the company says that a final concentration of $5 \mu\text{g } \mu\text{l}^{-1}$ ($0.5 \mu\text{l}$ in a typical $100 \mu\text{l}$ PCR) of Bind-Aid reagent is optimal. Bind-Aid is supplied in a ready-to-use form in 100- and 500- μl quantities for \$85 and \$179 (US), respectively, along with suggested protocols.

Shelf stockers

Human transferrin with a purity of greater than 99 per cent can now be obtained from HighBio (*Reader Service No. 108*). It is obtained from outdated plasma of volunteer blood donors after stepwise purification by polyethylene glycol precipitation,



High-purity human transferrin.

ion exchange chromatography and HPLC. Polymers that form during subsequent gamma irradiation are removed chromatographically. Each blood bag is tested individually for syphilis, hepatitis B and human immunodeficiency virus. HighBio says the biological quality of its human transferrin is tested under serum-free conditions on two lymphoblastoid cell lines and one hybridoma line. Transferrin in either an iron-free (apo-) or iron-saturated (holo-) form is available either lyophilized or as a sterile liquid combined with biosynthetic human insulin and selenium for direct use in cell culture.

Gibco BRL (Life Technologies) is featuring five new products for **neural cell culture**, which are designed to allow researchers to culture neural cells without having to pay custom-formulation prices (*Reader Service No. 109*). The lineup includes commercial versions of Bottenstein's G-5 and N-2 formulas, a modification of Brewer's B-18 formula, neural transmitter medium, and OPTI-MEM I reduced-serum medium. Gibco

BRL sells its products for neural cell culture as separate components to allow researchers to choose the basal media and supplement that is best suited to their application. And, as the media supplements are shipped as frozen concentrates, the company says the shelf life of the supplements is extended.

Bachem's 1992 **catalogue** provides a comprehensive listing of amino acid derivatives, resins, enzyme substrates and inhibitors, cytokines, growth factors, neurotoxins and other biologically-active peptides (*Reader Service No. 110*). Over 100 enzyme inhibitors and more than 600 substrates are featured, including acetyl pepstatin, inhibitors and substrates for cathepsin, angiotensin-converting enzyme and renin, chromogenic and fluorogenic substrates such as *p*-nitroanilides, β -naphthylamides and 7-amido-4-methylcoumarins. In addition, new fluorogenic HIV-protease substrates and inhibitors have been added as well as synthetic echistatin, which is chemically and biologically identical to the naturally occurring material. Echistatin, a toxic polypeptide obtained from the venom of the saw-scaled viper, binds tightly and specifically to the glycoprotein IIb/IIIa receptor on platelets, and competitively inhibits fibrinogen-dependent platelet aggregation induced by ADP, thrombin, adrenaline, collagen or platelet-activating factor. Bachem also offers a HIV-protease assay kit, crystallizable-grade HIV proteases, recombinant murine IL-10, and Sastrin amino acid resins for solid-phase peptide synthesis using Fmoc chemistry.

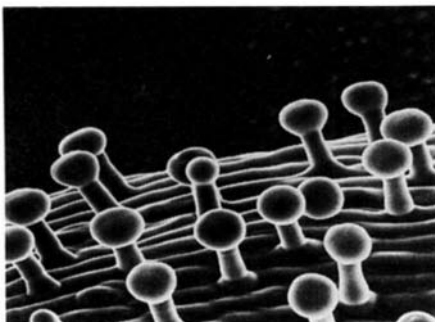
Limits of detection

Beckman has introduced the DU-600 series of **UV/visible spectrophotometers**, which combine advanced software and focused beam technology to provide detailed analysis of samples as small as two microlitres (*Reader Service No. 111*). The DU-640 model offers an extended wavelength range of 190 to 1,100 nm. In addition to absorbance and percentage transmittance, the instrument also can overlay 1st to 4th derivative and log absorbance. Scan speeds of up to 2,400 nm min⁻¹ are possible. Other standard features include

mathematical manipulation of scans, including peak and valley determination; addition, subtraction and multiplication; and background corrected peak measurements. And, if the appropriate sampling accessory is fitted, the DU-640 can perform kinetic calculation with simultaneous measurement of up to 12 samples. Data and methods are stored automatically in an internal nonvolatile memory and can be transferred to a 3.5-inch disk if an external data storage option is fitted. Additional software is available for single- and multi-component analysis, protein and nucleic acid determination, enzyme mechanism and inhibition determination, and gel scanning. The DU-650, an enhanced version of the DU-640, allows the user to tailor the software so that the instrument can perform specialized but routine tasks.

1992 Polaroid International Instant Photomicrography Competition

Prasium majus flower, $\times 300$, Steven Wagstaff, Ohio University, 2nd prize student micrography section, 1991 Polaroid International Instant Photomicrography Competition.



Cameras at the ready, Polaroid is calling for entries in the 1992 Polaroid International Instant Photomicrography Competition. Now in its eleventh year, a panel of photomicrography experts will award up to 30 prizes worth a total of \$13,600 (US) in six categories — electron micrographs of life science subjects on any Polaroid instant film, electron micrographs of materials science subjects on any Polaroid instant film, optical micrographs on any Polaroid instant black-and-white film, micrographs on any Polaroid instant colour print film, micrographs on PolaChrome instant colour slide film, and micrographs submitted by students on any Polaroid instant film. Entries must arrive at Polaroid no later than 10 July 1992. For more information, entry forms and the rules of the competition, fill in reader service 112, or contact Polaroid Corporation, 575 Technology Square-9P, Cambridge, Massachusetts 02139, USA.

The new **Lambda flow injection analysis (FIA)** system from Perkin-Elmer combines sensitive ultraviolet/visible spectrophotometric detection with automatic sample preparation (*Reader Service No. 113*). This set-up, the company says, should decrease analysis time and increase sample throughput. The system also has been designed to minimize the



Lambda flow injection analysis system.

amount of sample and reagent used and cut down on chemical waste. At the heart of the Lambda FIA system is the Lambda 2 UV/visible spectrometer, which has a wavelength range of 190 to 1,100 nm and which also can be used as a stand-alone spectrometer without any hardware changes. Other components of the system include the FIAS-200 pump, injection valve, chemifold, tubings and couplings, and an AS-90 autosampler that can accommodate up to 152 samples. The Lambda FIA system is controlled with Perkin-Elmer's flow injection analysis software, which not only controls the instrument and accessories, but also performs data evaluation.

Sample processing

Spectra-Physics offers a new **micro-robotic sample processor**, the AS3000, which is optimized for microlitre sample preparation procedures and injection onto an HPLC (*Reader Service No. 114*). Automated micro-volume sample preparation is designed to reduce the amount of solvent waste and eliminate the need to handle toxic solvents. In addition, sample throughput is increased and accuracy is improved — especially when working with microlitre volumes. The AS3000 is equipped with a built-in vortex mixer/heater and has a 120-sample vial capacity. According to the company, injection reproducibilities better than 0.5 per cent are standard.

In order to meet the requirement for time-saving systems for sample preparation in laboratories with large sample throughput, LDC Analytical has introduced PROSPEKT, an instrument for **programmable online solid-phase extraction** (*Reader Service No. 115*). This fully automated online sample clean-up and injection system combines the quality assurance advantages of a disposable cartridge system with the automated aspects of pre-column technology. Modular in design, the PROSPEKT system can be configured to suit several configurations. For example, a solvent delivery unit can be added, to select and deliver various solvents (up to 16 liquids) to the cartridge at constant flow rates and at high pressure. At the heart of the system is the PROSPEKT module, containing the cartridge transport and sealing mechanisms, and a powerful microprocessor to control all the modules. The disposable cartridges



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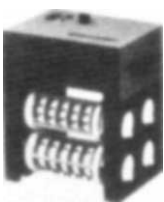
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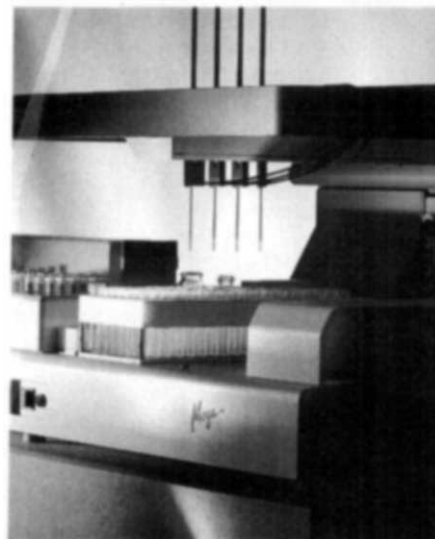
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are designed to suit HPLC conditions and are available from stock, containing a range of different phases (in 40 particle sizes). Specific materials can be packed with smaller particles upon request.

The new Proline electronic pipettors from Biohit Oy feature four operating programmes — pipetting, reverse pipetting, multiple dispensing and dilution (Reader Service No. 116). The pipettors, which can be programmed with variable pipetting speeds and small incremental volumes, use a d.c. motor for fast piston movements monitored by a real-time optical feedback system. This, together with microprocessor control, ensures reliable pipetting operations and eliminates the need for recalibration, says Biohit. Electronic operation combined with a lightweight and ergonomically designed operation button and tip ejector are designed to minimize stress and fatigue on both thumb and wrist. Two models are available: the 10–250- μ l and the 20–500- μ l versions. As a dilutor, they perform dilution ratios up to 1:25 and as a dispenser can attain dispensing routines such as $25 \times 10 \mu$ l, $25 \times 20 \mu$ l, $20 \times 25 \mu$ l, $10 \times 25 \mu$ l or $10 \times 50 \mu$ l with a single refill. The pipettors are supplied with a stand for storage when not in use; it also acts as a recharging station ensuring that the NiCd batteries are maintained at an optimal performance level at all times.

On show for the first time at Analytica, is the new MEGAFlex robotic sample processor from Tecan, which features 'intelligent' tip spacing, high-speed transfer, positive sample identification and versatile software (Reader Service No. 117). The four liquid-sensitive tips on the MEGAFlex are designed to transfer fluids accurately and reproducibly; tip spacing can be varied automatically to reach samples anywhere between 9 and 36 mm apart. Optimized washing procedures are designed to eliminate carryover; an optional 'fast wash' module cuts tip washing times still further, Tecan says.



Tecan's multi-tipped liquid handling system.

The robotic sample processor can accommodate as many as 160 13-mm tubes, four microplates plus reagent and sample bottles; an additional 256 sample tubes can be loaded by using the optional linear rack drive. Tecan eXecution software, which controls MEGAFlex, allows the user to implement pipetting sequences tailored to a wide variety of applications.

The IEC Centra-MP4 and refrigerated Centra-MP4R benchtop centrifuges from Life Sciences International cover a broad spectrum of applications from large batch/large volume spins to high-speed microcentrifugation (Reader Service No. 118). The capacity of the centrifuges can range from four 250-ml bottles through to 48 5-ml tubes, to up to 60 microtubes per run. The company says that 24 1.5-ml microtubes can be spun to 16,250 g in less than 13 seconds (combined acceleration/deceleration time) and, in the MP4R, at temperatures down to -5°C — even at full speed. A range of accessories is available for high-speed pelleting, density gradients, microplates and microscope slides. The SmartSet microprocessor control system controls all parameters — revolution, temperature and time to ± 5 r.p.m., $\pm 1^{\circ}\text{C}$ and ± 0 seconds, respectively, regardless of the selected speed or sample load. Up to 10 user-defined programmes can be stored in the memory. Other features include a spill-resistant membrane switch panel and a self-diagnostic capacity that alerts the user to operating errors. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. To obtain further details, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated. PCR is covered by patents issued to Cetus Corporation. AmpliTaq is a trademark of Cetus Corporation.

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Reader Service No.56

Jobs in the computer industry

Michael Dempsey

In spite of the recession, there is still room in computing for recruits with practical minds and an interesting background. Working in the vacation will pay off.

THE computer industry has always thrived on myths. Complex technology served to mystify the business world for the best part of a decade. Large companies parted with huge sums of money in return for systems they little understood. An act of faith was demanded by phenomenally profitable hardware companies and software houses. Buy this, you may not understand it, but believe us, it is very advanced.

This act was bound to falter. World recession coincided with a growing air of scepticism among the user community. The wall of technical jargon constructed around computer systems was breached by company accountants demanding to know what they were getting for their money.

A supremely confident industry found itself on the defensive. This sea change had a dramatic effect on staffing and recruitment. But to make sense of the new priorities, it is vital to keep the myths at bay.

Recruits

The computer business will always need fresh recruits. Experienced staff are valuable and hop between jobs in pursuit of generous salary increases. The industry wants to train graduates in particular company cultures while its customers are always on the look-out for information technology (IT) staff who can get the most out of their systems.

So the vacant posts are there. But no single qualification offers a route into programming or systems design. A computer science degree, as thousands of graduates discover every year, cuts no ice with industry. The fact is that many potential employers regard academic courses in IT with deep suspicion. They have a point. During the 1970s and 1980s, university courses on IT became notorious for a narrow and rigid focus. Computer executives regarded the products of such courses as heavily indoctrinated. Undoing this perceived bias takes time and money that employers would rather devote to creating an IT executive in their own image.

IBM selects the pick of each year's graduates and sets about turning them into corporate employees. Rivals deride this process as converting people into 'blue-suiters'. The biggest computer company in the world certainly sets store by a

rigorous company culture, but it also takes meticulous care in recruitment policy. A harsh economic climate has seen IBM contract from 383,000 employees in 1989 to 344,000 at the end of 1991. It still needs graduates, but they have to go a lot further to get an interview.

IBM's UK operation froze graduate



recruitment last year. It was a temporary measure, but has resumed at only 25 per cent of the previous level. A spokeswoman explained that one attribute is vital to join the new slimmed-down operation. "The sort of people we recruit are graduates, but not necessarily in a computing or science-related background. They have to work in groups, and, to be any good, they need communications skills."

Selling has become a hard task in the IT industry. One response is to push staff out into the marketplace, confronting a potential customer with more than the traditional salesman. Now the whole support team and backroom operation will roll up to convince the user that he can rely on a serious commitment from his computer supplier. In the case of IBM UK, this means that 70 per cent of the workforce now meet its customers. Stepping aboard as a £14,000-a-year trainee means being willing to act as a salesman. That takes confidence. The IBMer of the 1990s really must look the part.

Two IBM stars in the United Kingdom underline the fact that raw ability matters more than pertinent studies. Sir Tony Cleaver, the UK chairman, is a classics graduate. Nick Temple, recently appointed chief executive, is a software development engineer who joined IBM as a programmer straight from school.

Graduates are still in demand for IBM in the United States. During the 1980s, it hired around 3,000 every year. In 1991, only 1,700 were taken on. Although the current focus is on technical skills, US graduate recruits still need to mix with the business. "We're looking for people who work well with others, who can write well and communicate well," a spokeswoman explained. One significant detail about graduate selection in the United States is that IBM now "likes them to have some sort of pre-professional experience in a high-tech company". This is emerging as an unacknowledged international requirement.

Programmers in the United States can expect to begin work at a salary of between \$32,000 and \$36,000 a year. But the California sun is no longer an automatic benefit. After frantic growth in the 1980s, the West Coast's technology boom has stalled. Silicon Valley is reeling under the impact of recession while Southern California has been struck by defence cuts.

The Mid-West, South-East Texas and Florida are host to expanding industries and associated IT jobs. But the vacancies are in leading-edge technologies. The IT industry spawns a regular crop of acronyms and buzz-words to described the latest trends. Hands-on experience in these fields is what growing companies look for.

Computer-aided software engineering

(CASE) is an attempt to automate the labyrinthine process of writing and maintaining large computer programs. Expensive and sophisticated software tools characterize the onward march of CASE. They were sold on the back of intensive marketing in the late 1980s and any experience in making them work as promised is greatly appreciated.

Networking means linking terminals to get more value out of existing investments. It is related to client-server computing, a policy of carrying out processing locally on medium-sized computers rather than flashing everything back to costly mainframe computers. Both trends appeal to cost-cutting businesses.

The Graphical User Interface (GUI, touchingly pronounced Gooney) promises to turn every PC screen command into a series of graphics. GUIs are simple and promise to slash training costs for organizations with hundreds of PC operators. These trends all meet the current obsession with cost-cutting. A job-hunter identified with that aim becomes a desirable commodity.

Kenny MacIver is a senior research analyst with IDC Technologies. He spotlights GUIs as a boomtown for the shrewd graduates. "Eventually everyone who has written a program will have to change their applications to offer a GUI front-end. That means masses of work to be done." MacIver characterizes this task as "screenscraping", and advises graduates to take it seriously. "Get involved in a company that's moving its applications from a menu to a GUI-based screen display. Commercial organizations are waking up to the fact that this is going to be a huge job."

Catch-22

How does the aspiring graduate escape from the catch-22 that demands experience of IT work before opening the door to an IT career? Hunting down a slightly more demanding vacation job seems to be the answer. Any experience of work on a commercial computer project makes a tremendous impression on computer users and the industry that supports them. Nobody expects a student to be put in charge of implementing the payroll system for a multinational. But exposure to a commercial environment, with its relentless deadlines and scrutiny of spending, reassures the employer that here is a practically minded recruit.

Logica is a London-based systems consultancy with offices and operations across the globe. It enjoys a reputation as both an intellectually challenging company and one that is fun to work for. Competition for graduate places, reduced in the United States from 180 to 130 a year since the recession began, is fierce. But the successful applicants need more than a good degree. "We look for a reasonable academic

achievement, but they don't have to be first class. We try and get an interesting person, one that is keen to try things out and carry on learning," says Vernon Martinus, Logica's recruitment manager.

Starting salaries are between £14,000 and £15,300. The range is dictated by a points system. Postgraduate qualifications push a recruit up the scale, but so does vacation work. What counts for Martinus is that trainees have exerted themselves in the holidays. The company abandoned a formal graduate training programme after finding that this tended to isolate new arrivals from the business environment. It was replaced with a rolling scheme of short training modules that trainees attend between work on live projects. The aim is to force them into the commercial world as soon as possible, and any previous experience of coping with life on a project team is clearly a big bonus.

Logica's European offices have also cut back on recruitment. Its Dutch operation has halved the number of graduates taken on. Bull, the French computer manufacturer, has cut 5,000 jobs over the past year. Graduate recruitment continues, but with a new emphasis on the end result. Beyond predictable brave talk of a "lean and aggressive" company, Bull is trying its hand at the systems integration game. This means taking responsibility from the customer and installing a family of equipment from different sources. This in turn puts pressure on the staff to show real business acumen. "The people in our software and services arm need consultancy skills. They'll be competing with the big consultancies for business," Bull declares.

The Germans do not like to talk about recession. Tony Heimbring, international personnel director for Munich-based Softlab, is wary about the climate his software house finds. "We do not have a recession, but a time of more careful investment."

Heimbring echoes the need for some experience. "With a computer science degree, general opportunities are quite good, but only if you've invested the time to get in touch with the industry during vacations." Softlab operates at the leading edge of CASE technology. But however intricate the ideas are, Softlab has to sell them on to commercial customers. What Heimbring is looking for is someone who has gone beyond theoretical studies. A few weeks in another company "increases opportunities in a remarkable way".

Like Logica, Softlab operates a points system, boosting starting salaries by qualifications and previous practical experience. A first degree will earn £16,500. But add higher qualifications and evidence of relevant work and the Softlab novice can be paid £21,500. For Heimbring and his colleagues, recruits in the latter category bring a special benefit. "We want to see an understanding of the non-

technical aspects of the job, like how business planning is done, or what the customer looks for."

For job hunters who cannot offer industry experience, there are other ways to even the odds. Patrick Whale wears two hats in commenting on the recruitment market. A senior IT partner in the international management consultancy KPMG, he is also the current president of the UK Computing Services Association (CSA). He is reassuring on the general prospects — "the industry will never stop recruiting graduates" — but makes some frank observations. "We all have to face up to the fact that this industry isn't going to carry on upwards and onwards. A career in IT is no longer a safe option, and people should accept that."

Whale admits that CSA members share a reluctance to take on graduates with nothing more than a college course behind them. "People without work experience don't make much of a contribution at the start". And even if students have changed, some prejudices linger on. It is just as well to remind any prospective employer that you will make a good ambassador for the company. "In most cases a degree of smartness is important. The days when computing meant beards and sandals are gone!"

Coming across as someone who can look the part clearly counts. And some large companies offer a lucrative route to work experience for undergraduates. Digital Equipment (DEC) is second only to IBM in size. Yes, it has cut 20,000 jobs since 1989. But it still operates an industrial placement scheme for students. Successful candidates are sponsored through their degree and work with DEC during holidays. Not every student wants to sign up with the computer giant, but DEC will try to find for those that do not a position within its huge customer base. This is not an act of charity. Having worked for DEC and absorbed its practices, they will carry the company message to a wider audience.

Survival

If infiltrating the computer business begins to sound daunting, it's worth remembering the rewards. Ordinary project team leaders can expect to earn £35,000 – £50,000. IT directors, running the show for medium and large companies, are now often paid more than £100,000. The salary explosion of the 1980s has abated, but individuals who can trim down the IT budget are at a premium. That is probably the best way to view the world of IT. Under all the intellectual gloss, it is an industry surviving through a recession that has battered its customers. Interviewees who bear that in mind will start off on the right footing. □

Michael Dempsey is a freelance writer.

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